

DETERMINATION OF FIBER TYPE AND SIZE OF THE
OVINE QUADRICEPS MUSCLES AT DIFFERENT AGES

by

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INTRODUCTION

Although muscle sections may look relatively homogeneous when stained with hematoxylin and eosin, histochemical stains have revealed that there are different fiber types based on their preferred metabolic pathways.

To evaluate the response of a muscle to variety of pathological insults it is necessary to know the % of different fiber types, their mean diameter and the effect of age on each of these parameters. There is no reported data on the progressive change in either the % of different fiber types or mean fiber diameters with age in sheep.

Fiber determination in neonatal lambs has been done. Ashmore, 1970, named three fiber types, red, red and white. The semitendinosus, longissimus dorsi, psoas major, latissimus dorsi, gastrocnemius, serratus ventralis, supraspinatus, infraspinatus, semimembranosus, triceps brachii (longhead) and masseter muscles from six sheep have been histochemically examined by Suzuki, 1971^a, 1971^b, 1972 and fiber diameters and % fibers of different fiber types determined.

The quadriceps muscles have not been classified and this work was undertaken to provide base line data to evaluate pathological changes in ovine inherited muscular dystrophy. Fiber types were denoted as type I and type II_A and II_B a nomenclature first in human medicine (Brooke and Kaiser, 1970), and based on myosin adenosine triphosphate hydrolase (APTase), nicotinamide dinucleotide tetrazolium reductase (NADH-TR), and succinic dehydrogenase (SDH).

MATERIALS AND METHODS

Muscles samples 1 x 1 x 2 cm were excised from the middle of the vastus lateralis (Vl), vastus medialis (Vm), vastus intermedius (Vi) and rectus femoris (Rf) muscles from 15 sheep. Details of the number and age of sheep are in the Table I and II. Muscle samples were wrapped in gauze moistened with physiological saline solution (PSS) and held at 4° C for 1-12 hours. Muscle pieces 10 mm x 5 mm each were sliced with sharp razor blades, quenched in isopentane cooled to -90° C with liquid nitrogen. Serial sections 10 μ m thick were cut on a cryostat^a at -20° C, air dried for 30 minutes at room temperature and then stained with SDH (Nachlas, 1957), NADH-TR (Suzuki, 1971^a) and myofibrillar ATPase at pH 9.4 (Padykula and Herman, 1955). Other sections were preincubated in 0.2 M acetic acid-sodium acetate buffer at pH 4.3, 4.45 and 4.6 before the ATPase reaction to determine fiber type reversal and in 0.1 M glycine calcium formal at pH 7.0 to determine 3 distinct fiber types (Tunell, 1975).

Corresponding area of muscle stained in serial sections for ATPase and NADH-TR were photomicrographed at a magnification of 33x on the negative. Prints were enlarged 7x to give a final magnification of 231x.

Muscle fibers were classified as type I, type II_A and type II_B and the diameter of 200 fibers was measured across the greatest dimension of the lesser diameter in each muscle using a particle size analyzer^b. The mean and standard deviation of the fiber diameter of the fiber types was calculated for each muscle.

^a Ames Comp., Division of Miles Laboratory, Elkhart, Indiana.

^b Zeiss TGZ-3, Carl Zeiss, 7082 Oberkochen, West Germany.

RESULTS

The myofibrillar ATPase reaction at pH 9.4 routinely differentiated two fiber types, type I and II, but preincubation with Tunell's glycine buffered calcium formal (GBCF) revealed 3 fiber types (type I, II_A and II_B) in 75% of sections studied. Type I fibers stained light, II_A fibers darker and II_B fibers darkest (Figure 1). However, with this technique type II_A and II_B fibers could be differentiated at birth in only one of nine sheep. To confirm the classification of fibers II_A and II_B, serial sections stained by NADH-TR were examined. Type I fibers stained densely with diffuse granules, type II_A fibers stained with coarse granules concentrated beneath the sarcolemma and type II_B stained sparsely with large granules (Figure 2). Fibers (labeled type I_B) making up the vastus intermedius stained diffusely with coarser granules than the type I fibers (Figure 11). Staining results with the NADH-TR and SDH were similar, except that the tetrazolium deposits were heavier with the NADH-TR (Figures 3 and 4).

In young animals less than one year of age, differentiation between type II_B and II_A fibers based on both ATPase and NADH-TR was difficult, if not impossible. Muscle fibers in the youngest lambs (1-2 days old) were undifferentiated as judged by NADH-TR. In older sheep (2 years) good differentiation occurred with occasional problems differentiating type II_A from type II_B fibers with both ATPase and NADH-TR. Muscle sections which were equivocal because of inconsistent staining were discarded and not included in estimation of fiber diameters or % fiber types.

Acid preincubation using acetate buffers produced reversal of the type I and type II fibers but was not as effective in delineating three fiber types as the GBCF technique (Figure 1). Preincubation at pH 4.45 and 4.6 produced reversal and many times revealed three fiber types; however, the medium staining fibers showed variation in staining intensity so that trouble classifying the fibers occurred. The type I_B fiber stained densely and diffusely with NADH-TR and after preincubation at pH 7 in GBCF just slightly lighter than the type II_A fiber (Figure 5).

Three of the four muscles of the quadriceps are mixed fiber type muscles with the Vi being the exception. It consisted entirely of type I_B (Figures 8-12) fibers in nine of 12 sheep and was only mixed fiber muscle in sheep less than 2 years of age.

Details of fiber diameter and proportion of fiber type in each muscle are given in Tables I and II. Type I fibers in the Vl, Vm and Rf increased in diameter from neonates to two years after which time no significant change was found. A similar increase occurred in the type II fibers considered as a group or separated into types II_A and II_B. The proportion of type II fibers decreased from 85-90% in the Rf, Vl and Vm in neonates to 70-80% in adult sheep as the type I fibers increased.

One subgroup of type I fiber designated as type I_B were identified twice in the Rf and once in the Vm (Figures 5-7). However, the mean diameter of this fiber in the Rf (66.8 and 60.8 μ m) and Vm (70.4 μ m) was larger than the mean of the similarly designated fibers of the Vi (51.4 μ m). In the Rf and Vm where the type I_B fiber was present, type II fibers could not be divided with certainty into type II_A and type II_B with either NADH-TR or ATPase.

DISCUSSION

Because different fiber types respond differently to different insults of disease, it is necessary to have basic data from normal muscle for evaluation of change in diseased muscle. This study was undertaken to establish the percentage of different fiber types and their mean diameter in the quadriceps muscle of sheep, so that the pathological changes in ovine inherited muscular dystrophy could be evaluated. Classifying and measuring fibers is time consuming, but can be done reasonably if the 3 fiber types are visible. Preincubation in Tunell's GBCF (1974) revealed this staining in 75% of ovine muscle from all ages, always possible in 5 year old sheep. Where type II_A and II_B fibers were not differentiated, photomicrographs of serial sections stained with NADH-TR were examined to distinguish these 2 muscle types. Measurement of muscle fiber diameters is done quickly by the particle size analyzer^a and the measurement of the maximum diameter across the lesser aspect of the fiber has been shown to be reliable even if the section is tangential and not exactly transverse (Dubowitz and Brooke, 1973).

Reports indicate no difficulty in obtaining reliable results with the myosin ATPase reaction in the dog (Cardinet, 1971), horse (Linholm, 1974) (Aberle, 1976), rat (Padykula and Herman, 1955) and mouse, guinea pig, cat (Dubowitz, 1968). Of the 60 muscles sampled from the ovine quadriceps, 52 could be divided into a type I and II classification. Lack of identification in 8 samples was due to artifact due freezing or thawing and poor staining, and these were discarded. Collecting muscle

^aZeiss TGZ-3, Carl Zeiss, 7082 Oberkochen, West Germany.

immediately after euthanasia and holding it in saline moistened gauze for no longer than 4 hours provided the best results. The use of fresh $(\text{NH}_4)_2\text{S}$ for precipitation of the cobalt ion in the ATPase staining has been suggested as a necessary adjunct for good staining (Suzuki, 1975).

Acid preincubation of ovine muscle did not always delineate three fiber types as reported in human muscle (Dubowitz and Brooke, 1973) (Brooke and Kaiser, 1970) and in rat muscle (Samaha et al, 1970). The fiber reversal for type I and type II fibers was complete at pH 4.3 (Figures 5 & 6) but differentiation into type II_A and type II_B fibers was difficult with many gradations of stain making reliable classification impossible. Temperature stabilization has been reported as necessary for consistent results with acid preincubation (Kahn, 1974) but it was not used in this study.

The SDH stain is specific for a mitochondrial enzyme whereas NADH-TR stain also stains the sarcoplasmic reticulum (SR) and other intermyofibrillar substances (Dubowitz and Brooke, 1973). In the sheep these stains produced similar results as previously described (Suzuki, 1971), the NADH-TR producing a darker concentration of staining thus being easier to photograph for fiber typing than the SDH reaction (Figures 3 & 4).

The proportion of fiber types varies in the same muscle in different sheep and in different muscles in the same sheep (Suzuki, 1971^a, 1971^b). The biceps and vastus lateralis muscles of humans normally have a ratio of 1:2 in type I to type II fibers. The gluteus medius of trained Standardbred horses was found to have 24% type I, 54% type II_A and 22% type II_B fibers (Lindholm, 1974).

The semitendinosus from 2 year old ponies showed 31% type I, 40% type II_A and 29% type II_B fibers (Aberle, 1976). Care was taken in

these studies to remove samples from the same site as in this study since different parts of muscles have different proportions of fiber types. The high proportion of type II fibers 90.4% in the RF, 86.0% in the VI and 85.9% in the Vm at birth changed to 71.8%, 73.7% and 71.7% respectively indicating a transformation of fibers to type I fibers or loss of type II fibers.

The Vi of the sheep has been described as being entirely composed of a strong NADH-TR reactive fiber showing a strong ATPase reaction after preincubation at 4.3 (Suzuki, 1975). This makes the fiber a subclass of the type I fiber and has been called a type C fiber rather than a type D or E in Suzuki's classification (Suzuki, 1975). This study shows this fiber to be slightly stronger than the type I fiber using GBCF at pH=7.0 preincubation for ATPase. This fiber we have called the type I_B fiber stains similarly to the type I fiber for ATPase after 4.3 pH preincubation. Type II fibers were seen in sheep under 2 years of age (Figures 15 & 16) but never in adult sheep, perhaps indicating a transformation of the type II fibers into type I fibers as suggested in the change in proportion of the other muscles of the quadriceps.

Some muscles are predominately or completely of type I or NADH-TR intermediate staining fibers such as the soleus muscle of the guinea pig (Barnard, 1971), cat (Dubowitz, 1968) and rat (Edgerton, 1968) and in the masseter of the sheep (Suzuki, 1972). Subdivision of the type I fiber or NADH-TR intermediate fiber (Barnard, 1971) has been completed only in sheep where slight differences in the SDH, NADH-TR, phosphorylase and ATPase staining have been described by Suzuki, 1971^a, 1971^b, who called them C, D, and E fibers. The type C fiber has a diffuse dense stain with SDH and NADH-TR, weak ATPase, weak phosphorylase and weak to

moderate glycogen reaction (Suzuki, 1971^b). The type D fiber is a larger fiber than the type C having a high NADH-TR activity, low phosphorylase, low in ATPase and always low in glycogen (Suzuki, 1971^b). The type E fiber described as the only fiber type in the ovine masseter muscle (Suzuki, 1972) shows an intense NADH-TR and SDH stain being more marked than any other type I fiber. The Vi from sheep in our study have fibers (I_B) fitting Suzuki's type E fiber having a denser staining than the type I fiber with NADH-TR. The large I_B fiber found twice in the Rf and once in the Vm on the other hand would classify as a type D fiber using Suzuki's classification being large fibers similar to the type C staining and described in the muscles of the pectoral girdle (Suzuki, 1971^b).

Differentiation of muscles into 2 fiber types occurs at birth in the rat, rabbit, hamster, guinea pig (Dubowitz, 1968) and in the sheep, pig, cow (Ashmore, 1971, 1972). The differentiation in sheep into type I (red), II_A (red) and II_B (white) fibers has been reported as difficult in neonatal sheep (Ashmore, 1971). The cat and rabbit showed better differentiation into type I and type II fibers with the ATPase method (Dubowitz, 1968) after the first week of life similar to our findings in lambs. Complete differentiation into three fiber types appears more difficult in the rat, rabbit and hamster with the oxidative enzymes (Dubowitz, 1968) and in lambs (Ashmore, 1971) but not in humans or the guinea pig (Dubowitz, 1968). In our experience lamb muscle at 1-2 days of age was soft, hard to cut and orient for sectioning causing the poor results in classification at this age (Figures 12, 13 & 14).

Mean fiber diameters changed significantly after birth as was expected. Mean fiber diameters in previous studies of sheep muscles (longissimus dorsi, semitendinosus, psoas major, latissimus dorsi,

gastrocnemius, serratus ventralis, suprapinatus, infraspinatus, semi-membranosis, and triceps brachii) in 3 adult sheep (age not given) were smaller than those recorded from the quadriceps group of muscles from adult sheep, over 2 years of age. The fibers have ceased growing in sheep older than 2 years since no significant difference was found between the 2 and 5 year old sheep. The comparison of type I versus type II fibers by mean diameter should not be used as a measure of fiber size since the type II_A fibers are smaller and the type II_B fibers larger than the type I fibers. The cumulative average of the type II fibers may therefore be close or equal to the mean fiber diameter of the type I.

Pathological changes in muscle affect the fiber diameter and proportion. Starvation causes atrophy or lessening of all fiber type diameters (Suzuki, 1973). Denervation will cause a decrease in diameter of all fiber types in a muscle fascicle whereas spinal problems with loss of specific motor neurons cause atrophy in one type of fiber (Dubowitz and Brooke, 1973). Disuse atrophy shows a decrease in the diameter of type II fibers and the training effect causes hypertrophy of the type II fibers (Dubowitz and Brooke, 1973) and a proportional increase in type II_A fibers (Linholm, 1974). Muscular dystrophies produce decreases particular fiber type diameters and cause a change in proportion resulting in a fiber type predominance or fiber type deficiency (Dubowitz and Brooke, 1973). Knowledge of the normal fiber diameter and proportion of fiber types will allow for diagnosis and classification of muscle disease in the sheep and specifically to compare the changes of ovine inherited muscular dystrophy (McGavin and Baynes, 1969) to the normal muscle.

SUMMARY

The fiber size and proportion of fiber type of the quadriceps muscles from 15 sheep, ranging from 1 day to 5 years of age were classified using myosin adenosine triphosphatase and nicotinamide adenosine dinucleotide-tetrazolium reductase stained sections. The vastus lateralis, vastus medialis and rectus femoris were mixed muscles containing three fiber types (I, II_A and II_B). The vastus intermedius contained predominately a type I like fiber.

Fiber diameter increased from 15.8 to 47.0 um in the vastus lateralis, 15.6 to 50.7 um in the vastus medialis, 17.5 to 46.5 um in the rectus femoris and 26.7 to 51.8 um in the vastus intermedius from birth to 5 years. The proportion of type II fibers decreased from birth to two years of age in the vastus lateralis, vastus medialis and rectus femoris. The vastus intermedius contained 100% type I like fibers in sheep 2 years and older.

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TABLE I

Muscle	Age	No. of Sheep	% All Muscle Fibers Type I Not Including I _B	Mean Diameter Type I Fibers (um)	S.D.	% All Muscle Fibers Type I _B	Mean Diameter Type I _B Fibers (um)	S.D.
Rectus Femoris	5 yr	6	28.2	46.5	10.40			
	5 yr	1	39.8	43.8	6.95	26.0	66.1	8.14
	2 yr	1	23.4	45.2	10.01			
	2 yr	1	24.9	43.8	11.509	23.5	60.8	11.89
	7 mo	2	29.1	35.1	6.45			
	4 mo	2	20.4	25.7	6.83			
	10 day	1	14.9	19.5	3.86			
	1-2 day	3	9.6	17.5	3.27			
Vastus Lateralis	5 yr	6	26.3	47.0	11.4			
	2 yr	2	20.1	43.2	9.49			
	7 mo	2	18.5	35.3	6.77			
	4 mo	1	13.4	26.4	5.74			
	1-2 day	3	14.0	15.8	2.58			
Vastus Medialis	5 yr	5	23.3	50.7	8.45			
	5 yr	1	23.9	45.6	9.13	9.6	70.4	13.34
	2 yr	2	28.8	43.6	7.93			
	7 mo	2	27.9	37.4	7.29			
	4 mo	2	27.0	26.1	6.32			
	1-2 day	2	14.1	15.6	2.03			
Vastus Intermedius	5 yr	5				100.0	51.8	8.35
	2 yr	1				100.0	49.1	6.95
	7 mo	1				88.0	48.7	5.89
	4 mo	1				96.4	38.0	6.69
	4 mo	1				100.0	46.8	7.33
	10 day	1				100.0	29.7	3.40
	1-2 day	1				94.3	26.7	3.32

TABLE II

Muscle	Age	No. of Sheep	% Muscle Type II	Mean Diameter All Type II (um)	S.D.	No. of Sheep	% All Muscle Type IIA	Mean Diameter Type IIA (um)	S.D.	No. of Sheep	% All Muscle Type IIB	Mean Diameter Type IIB (um)	S.D.
Rectus Femoris	5 yr	6	71.8	44.5	9.22	6	30.1	41.3	9.07	6	41.7	46.8	8.14
	5 yr	1	34.1	39.2	8.90	0				0			
	2 yr	1	76.6	45.8	13.8	1	33.2	35.2	12.67	1	43.4	54.0	15.38
	2 yr	1	51.6	39.5	9.03	0				0			
	7 mo	2	70.9	36.2	8.36	1	51.3	28.5	6.88	1	24.7	35.2	8.94
	4 mo	2	51.6	26.8	6.08	1	5.7	20.5	6.19	1	75.0	27.3	5.75
	10 day	1	85.4	22.8	5.38	0				0			
	1-2 day	3	90.4	17.0	4.13	0				0			
Vastus Lateralis	5 yr	6	73.7	44.6	11.8	6	28.3	40.4	11.9	6	45.2	47.8	18.2
	2 yr	2	79.9	41.3	9.46	1	39.9	44.7	8.60	1	40.4	59.7	11.09
	7 mo	2	81.5	32.4	7.72	1	51.7	31.5	5.99	1	26.9	41.5	6.96
	4 mo	1	86.5	31.5	7.66	0				0			
	1-2 day	3	86.0	16.1	3.44	1	71.0	12.6	3.13	1	11.8	14.9	2.73
	5 yr	5	71.7	44.0	8.72	5	35.7	41.0	9.07	5	36.0	45.3	7.89
	5 yr	1	65.0	36.5	8.42	0				0			
	2 yr	2	76.2	39.8	10.74	2	25.7	34.6	8.24	2	51.0	42.4	10.6
Vastus Medialis	7 mo	2	72.1	42.1	7.82	1	39.8	39.6	5.58	1	30.3	47.0	6.78
	4 mo	2	73.0	26.4	6.61	1	21.6	21.6	4.75	1	48.9	26.3	4.53
	1-2 day	2	85.9	19.8	4.20	0				0			
	5 yr	5	0			0				0			
	2 yr	2	0			0				0			
	7 mo	1	12.0	41.9	6.89	0				0			
	4 mo	1	3.8	34.5	4.89	0				0			
	4 mo	1	0			0				0			
Vastus Intermedius	10 day	1	0			0				0			
	1-2 day	1	5.7	24.5	3.6	0				0			

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Figures 1 and 2: Serial sections from Rf, 5 year old ewe.

Figure 1 - GBCF ATPase X231. Notice type I light staining fibers (I), type II_A medium staining fibers (II_A) and type II_B dark staining fibers (II_B).

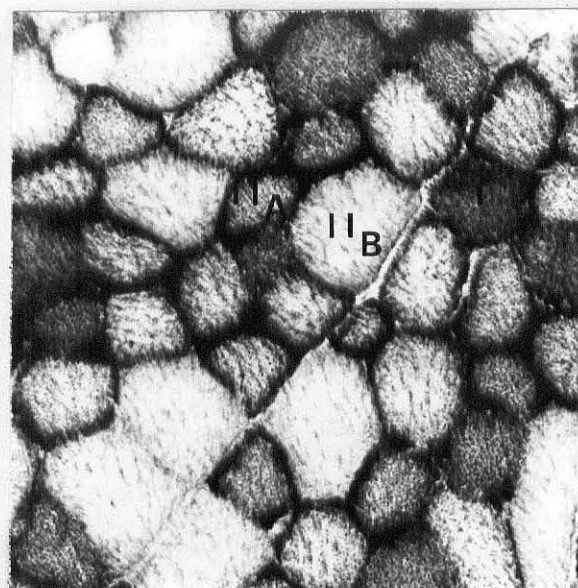
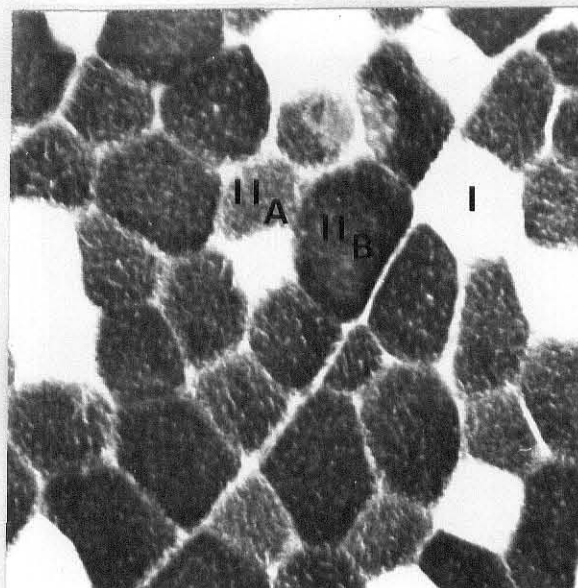
Figure 2 - NADH-TR X231. In type I fibers (I) tetrazolium stain is dense and diffuse, type II_A (II_A) fibers have dense subsarcolemmal staining and type II_B (II_B) fibers have sparse intermyofibrillar stain.

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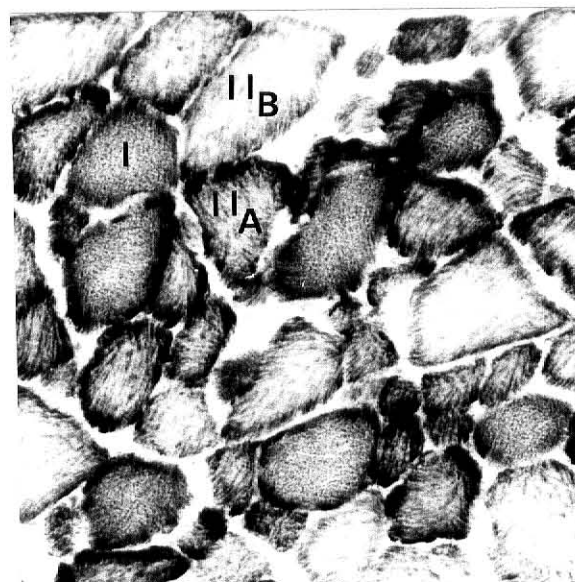
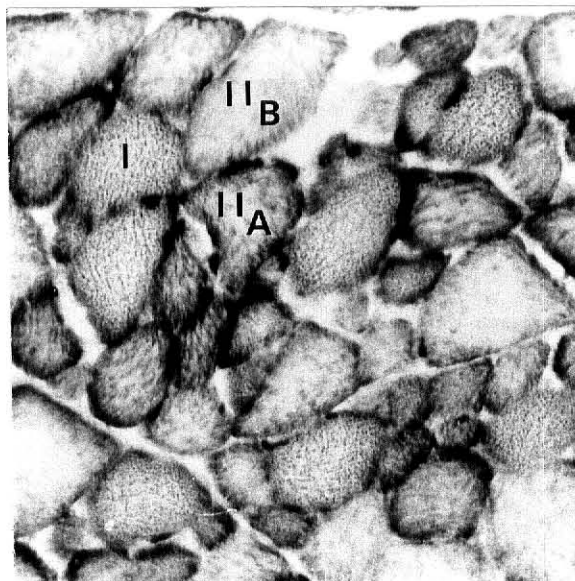
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Figures 3 and 4 - Serial sections from the V1 in a 4 month old lamb stained with SDH X231 (Fig. 3 TOP) and NADH-TR X231 (Fig. 4 BOTTOM). Notice staining is similar for I, II_A and II_B fibers but more dense with NADH-TR (Fig. 4).

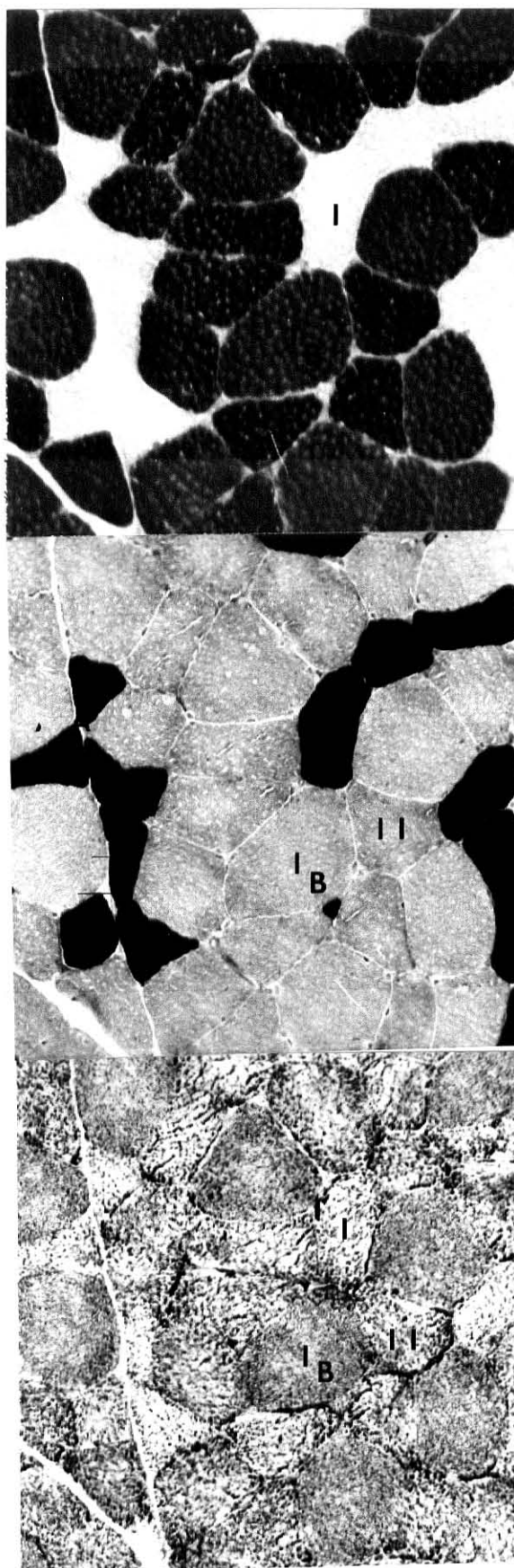


Figures 5-7: Serial sections from the Rf, 5 year old ewe.

Figure 5 - GBCF ATPase X231. Notice that only two fiber types, type I and type II are differentiated with no subdivision of type II fibers into type II_A and II_B. Type I_B fibers are larger than the type I or type II fibers.

Figure 6 - Preincubated at pH 4.3 ATPase X231. There is reversal of staining of both type I and type II fibers.

Figure 7 - NADH-TR X231. The type I_B fibers have a greater diameter and diffuse tetrazolium staining.



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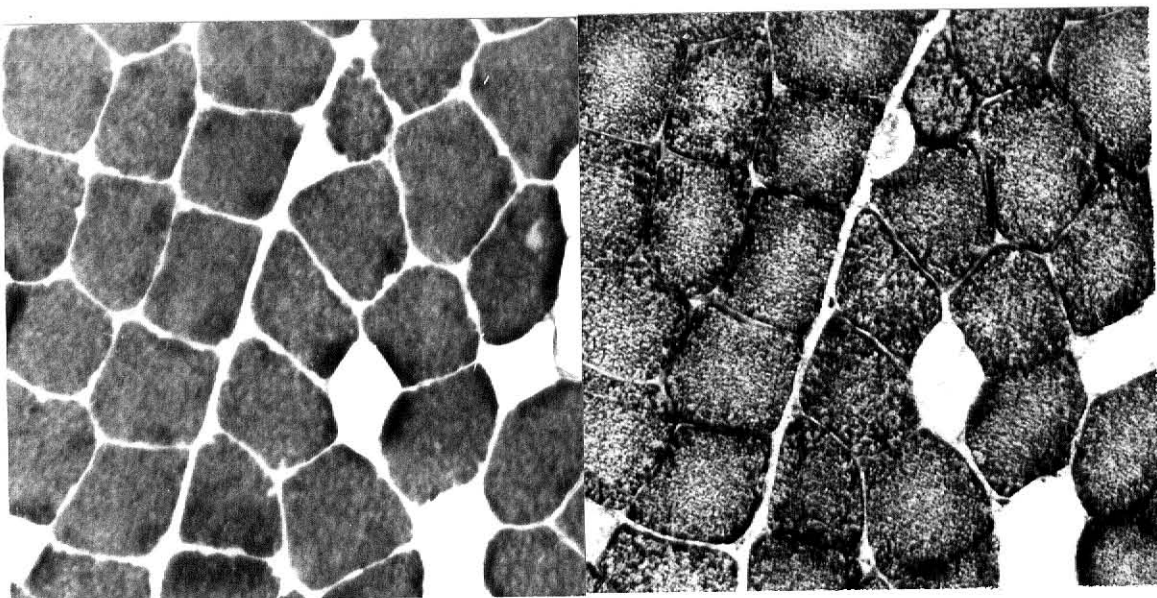
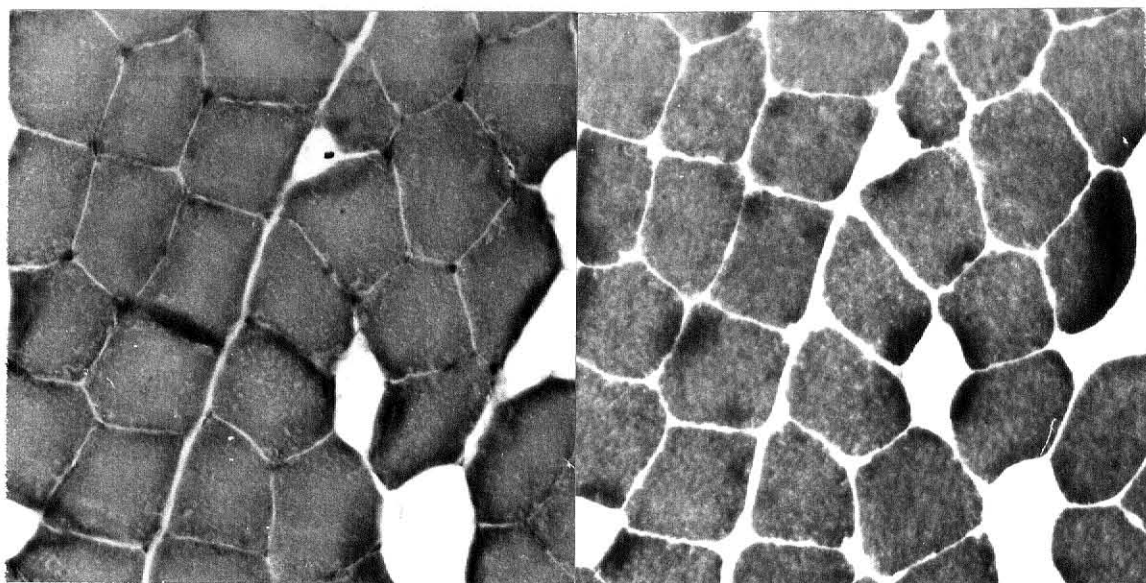
Figures 8-11: Serial sections from the Vi, 5 year old ram.

Figure 8 - GBCF ATPase X231. Notice uniform moderate staining of the type I_B fibers (LEFT).

Figure 9 - Preincubation at pH 4.45 ATPase X231. Staining is uniform and dark (RIGHT).

Figure 10 - Preincubation at pH 4.3 ATPase X231. Notice uniform dark staining as seen at the 4.45 pH preincubation (LEFT).

Figure 11 - NADH-TR X231. Notice dense and diffuse intermyofibrillar staining characteristic of a type I fiber (RIGHT).



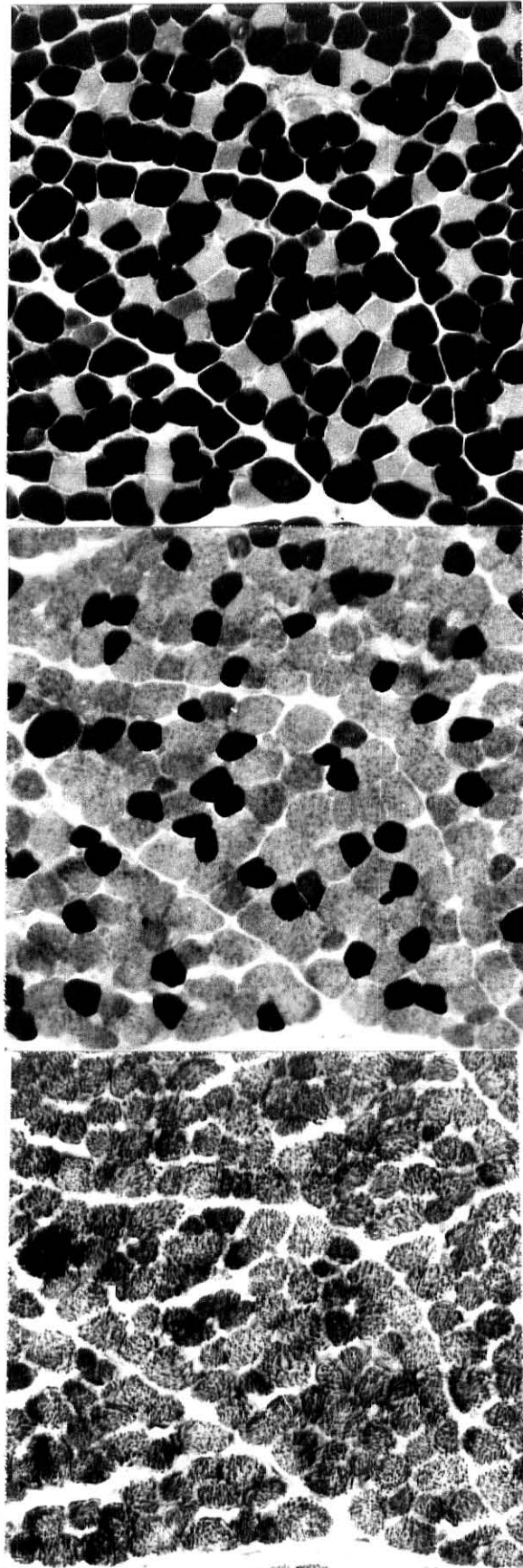
24.

Figures 12-14: Serial sections of the V1 from a 1-2 day old lamb.

* Figure 12 - GBCF ATPase X231. Notice the difficulty in differentiating the dark staining fibers into II_A and II_B.

Figure 13 - Preincubated at pH 4.3 ATPase X231. Notice the complete reversal of type I and type II fibers.

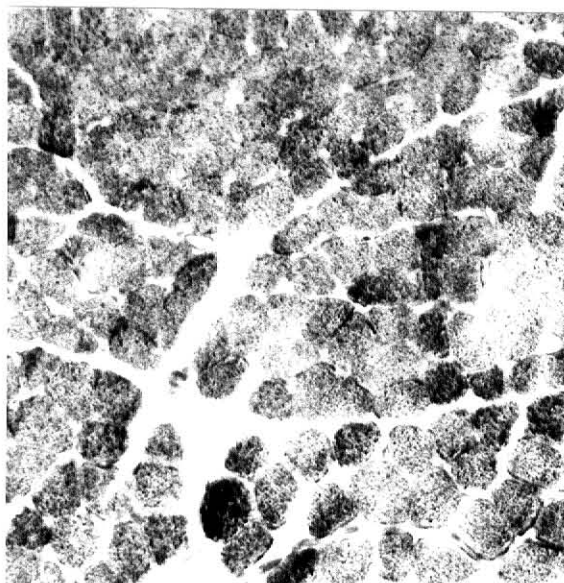
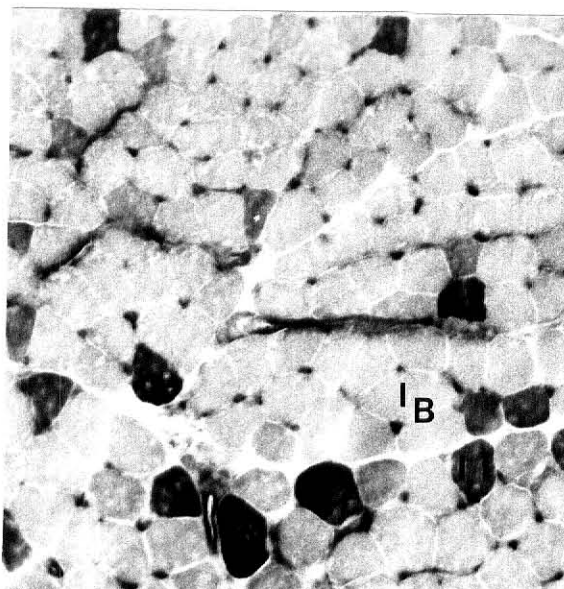
Figure 14 - NADH-TR X231. Notice differences in density of the tetrazolium staining but not enough differentiation to delineate type I, II_A and II_B fibers.



Figures 15 and 16: Serial sections of the Vi from a 1-2 day old lamb.

Figure 15 - GBCF ATPase X231. Notice the few dark staining type II fibers surrounded by a majority of the type I_B fibers. Accurate differentiation into type II_A and II_B is not possible.

Figure 16 - NADH-TR X231. Notice the uniform dense tetrazolium staining which does not allow determination of fiber types.



ACKNOWLEDGEMENTS

The author wishes to express his appreciation to M. D. McGavin for his assistance in completing this project and thesis. Thanks also to J. E. Smith for his help with the analysis of the data and to D. H. Kropf for his help in obtaining sheep for the project.

APPENDIX

LITERATURE REVIEW

Historical Background

Visual recognition of red and white muscle was recorded as early as 1687 by Stefano Lorenzini (Ciacco, 1898) (Dubowitz and Brooke, 1973). Their differences in speed of contraction and the blood supply were also recognized at an early date (Ranvier, 1873, 1874). The physiological response was correlated with the different colored muscle fibers. Red muscle, such as in ocular muscles or the muscles of mastication, usually had the role of more continuous activity (Knoll, 1891). Based on mitochondrial and fat content (Knoll, 1891), other muscles were found to be mixed fiber types.

Histochemical Techniques

Histochemical staining added a new dimension to the study of muscle. These techniques are based on the marking of an enzymatic reaction in a histologic section. Two classifications have been developed based on a hydrolytic reaction system such as adenosine triphosphate hydrolase (ATPase) splitting off the terminal phosphate from ATP residing in myosin or sarcoplasmic reticulum (Pearse, 1968), and (2) a change by electron transfer (Verity and Coleman, 1973) such as oxidation of nicotine adenine dinucleotide via a tetrazolium reductase (NADH-TR) (Dubowitz and Brooke, 1973).

The original method for the ATPase reaction (Gomori, 1941) was found more sensitive on frozen sectioned tissue than on paraffin-fixed tissue. In this test, most sensitive in the alkaline range of 9.2-9.4 pH (Padykula and Herman, 1955), a section of muscle is incubated in a

buffered calcium solution which allows precipitation of CaPO_4 at the site of hydrolysis of the terminal phosphate group from ATP. A stain precipitate is then produced by a cobalt exchange for the calcium and subsequent precipitation of cobalt with $(\text{NH}_4)_2\text{S}$. This method is reported to stain the myofibrillar portion of the muscle fiber at pH 9.4 (Guth and Samaha, 1969) and the intermyofibrillar substance of the fiber at pH's below 5.0 (Brooke and Kaiser, 1969). The intermyofibrillar network is made up of the mitochondria, sarcoplasmic reticulum, transverse tubular system, and aqueous sarcoplasm (Brooke and Kaiser, 1974). Two types of fibers, I and II, are readily determined using the 9.4 pH incubation and three types, I, II_A and II_B , can be seen by using several acid preincubation before the incubation at pH 9.4 (Brooke and Kaiser, 1970). Preincubation in buffered formalin also produces 3 fiber types (I, II_A , II_B) (Table I) (Guth and Samaha, 1969) (Tunell, 1975).

A method for succinic dehydrogenase staining was reported in 1935 (Semenoff, 1935) using methylene blue as the indicator. Later tetrazolium dye was used as a hydrogen acceptor making differentiation of types I, II_A and II_B possible (Table I) (Seligman and Rutenburg, 1951) (Padykula, 1952). Nitro BT, a form of tetrazolium, was also used for a simplified method to produce the SDH and NADH-TR stains (Nachlas, 1957). SDH, which is only present in the mitochondria at the site of formation of fumarate from succinate, can pass the H^+ to the cytochrome oxidase system without the use of coenzymes (Dubowitz and Brooke, 1973). Whether an intermediate enzyme is necessary to pass the H^+ on to reduce the tetrazolium and produce a blue dye is not known. It is thought that this reaction requires less intermediates, making the staining of the mitochondria more accurate than other stains (Dubowitz and Brooke, 1973).

The density of the SDH stain is therefore used to separate fiber types on the basis of concentration of mitochondria (Padykula, H. A., 1952).

In the NADH-TR reaction a coenzyme transfers H^+ 's from reactions in the mitochondria and the sarcoplasm. This was early called DPNH-diaphorase (Ogata and Mori, 1964) and is thought to use a tetrazolium reductase to pass the H^+ to the Nitro BT (Dubowitz and Brooke, 1973). The NADH-TR reaction is considered similar to the SDH reaction staining moderately (Ogata and Mori, 1964) or more dense (Dubowitz and Brooke, 1973) (Suzuki, 1971) in comparison, staining the sarcoplasmic reticulum as well as the mitochondria.

Other oxidative enzymes used for histochemical analysis are lactic dehydrogenase (LDH) and alpha-glycerolphosphate dehydrogenase (α GPD). LDH is a soluble enzyme which because of its diffusion throughout the incubating solution and tissue section is not considered a dependable test since the site of the stain may not be staining the actual site of enzyme reaction. Even though the site of staining of the SDH and NADH-TR are still not certain (Brooke and Kaiser, 1974), the site of both LDH and α GPD staining are even less reliable since the NADH-TR enzymes are necessary for the final H^+ transfer to the tetrazolium dye. These reactions therefore stain in a similar pattern to the NADH-TR (Ogata and Mori, 1964) (Dubowitz and Brooke, 1973).

Phosphorylase is also used as an indicator of glycolytic potential of the muscle fiber by producing glycogen from glucose-1-phosphate, the reverse of the normal use of the enzyme in the viable muscle. A dilute iodine solution stains the starch that has been produced (Takeuchi and Kuriaki, 1955) with color dependence on the length of hexose chains (Dubowitz and Brooke, 1973). The PAS stain can also be used to stain

the results of the phosphorylase reaction staining native glycogen and the newly formed glycogen, whereas the iodine can only stain the newly formed glycogen (Cardinet, 1971).

The PAS stain indicates 1:2 glycol groups which have been oxidized by periodic acid and marked with Schiff's reagent (Dubowitz and Brooke, 1973) but this staining does not always correspond with the results of the phosphorylase stain (Dubowitz and Brooke, 1973).

Histochemical Fiber Classification

The classification of muscle fibers has become important to the physiologist, animal scientist and physician. Workers in these areas have used different nomenclatures to describe the division of muscle fiber which has resulted in considerable confusion. In Table II the different nomenclatures are compared.

The oxidative enzymes were used first with fish, frog, bird, and mammalian in which three fiber types were described--a large white fiber with sparse staining, a red fiber with dense staining and a medium fiber with intermediate staining (Ogata, 1958). Human muscle was first investigated using SDH on autopsy specimens where smaller fibers were shown to have more reactive staining. A reciprocal staining was found between oxidative enzymes and phosphorylase in warm-blooded mammals (Dubowitz, 1968) and humans (Engel, 1962). This is not universally true as demonstrated in sheep muscle (Suzuki, 1971). A nomenclature was produced on the reciprocity of these enzymes in the rat naming fibers low oxidative, high glycolytic and high oxidative, low glycolytic (Dubowitz and Pearse, 1960).

Another classification was based on ATPase staining. Type I fibers stained lightly and type II fibers stained dark (Engel, 1962). Alkaline

and acid preincubation has been used to differentiate the myofibrillar and intermyofibrillar ATPase producing three fiber types with the ATPase method (Guth and Samaha, 1969) (Samaha, et al, 1970) (Brooke and Kaiser, 1970) (Guth and Yellin, 1970). Work with ovine (Ashmore, 1972) (Suzuki, 1971^a, 1971^b, 1972) and bovine muscle (Ashmore, 1972) introduced two more nomenclatures where different names were given to same fiber based on mitochondria content determined by oxidative stains. Though the nomenclatures are confusing, they can be compared as shown in Table II. The same type of muscle fiber will have variation between staining intensity and density between species (Guth and Yellin, 1971) (Brooke and Kaiser, 1974). This also applies to the same muscle fibers in the same species between different muscles (Brooke and Kaiser, 1974). When both the ATPase stains are used to make a classification, extra types of fibers have been described to account for the lack of reciprocation between the ATPase and NADH-TR staining except when using an acid preincubation with ATPase staining (Brooke and Kaiser, 1974). Some workers have attempted to define more than three basic fiber types, I, II_A and II_B; eg. Type II_C fiber defined by ATPase (Dubowitz and Brooke, 1973) and divisions of the type I fiber as C, D & E (Suzuki, 1971^b, 1972, 1975).

Physiologists tried to classify the fibers by naming the fiber according to their function and metabolism, fast twitch glycolytic, slow twitch oxidative, and fast twitch oxidative-glycolytic (Peter, 1972) and slow twitch, fast twitch and fast twitch high oxidative (Lindholm and Piehl, 1974). Stimulation of single nerve fibers provided proof that the qualitative staining with ATPase or oxidative enzymes actually correlates with muscle fiber function (Barnard, et al, 1971) (Burke, 1971). That assumption is based on the physiologic responses of muscles made up of

type I fibers such as the soleus in the guinea pig (Barnard, et al, 1971) and the cat (Henneman and Olson, 1965).

Contraction time is directly related to the ATPase activity of myosin (Barany, 1967). Muscles having higher numbers of high ATPase activity fibers have higher speeds of contraction (Burke, R. E., et al, 1971).

The use of the terms "red", "white" and "intermediate" nomenclature causes confusion since the "white" fiber has been labeled type II and the red fiber type I (Dubowitz and Pearse, 1960). The type II fibers, however, include red and white fibers which were later divided with acid preincubation before ATPase staining (Brooke and Kaiser, 1970) (Guth and Samaha, 1969). Electron microscopy has supported the basic three fiber types (Gauthier, 1971). The type II_A, or "red", fiber has large numbers of subsarcolemmal mitochondria with closely packed cristae, as well as rows of large mitochondria in between the myofibrils. The type I fiber, or "intermediate" fiber, resembles the type II_A fiber but has less mitochondria in the subsarcolemma aggregations and the cristae are not as dense. The Z lines are also thinner than in the type I_A fiber. The type II_B, or "white", fiber has sparse numbers of mitochondria and are noticeably absent from the intermyofibrillar spaces. Its Z line is the thinnest of all three fibers (Gauthier, 1971). The configuration of the sarcoplasmic reticulum also differentiates the three fiber types providing an elaborate network of tubules in the red fiber at the H band with broad parallel tubules in the white fiber (Gauthier, 1971). This provides a greater membrane surface in the red fiber.

Uses of Histochemistry

Histochemistry has been used in laboratory investigation to help define the function of muscles and the motor neuron. The classification of muscles as to the percentage of fibers can be associated with twitch time and contractile properties (Barnard, et al, 1971) (Henneman and Olson, 1965).

Animal scientists have used histochemistry of muscle as a tool to study the development of muscle in relation to weight gain and tenderness of meat (Davies, 1972) (Holmes and Ashmore, 1972) (Ashmore and Doerr, 1971) (Ommer, 1971) (Suzuki and Tarmate, 1974) (Suzuki, 1972², 1972, 1975).

Clinical investigation has used histochemistry for diagnosis and research in medicine. Muscle biopsies in humans have led to normal values of the sizes and proportions of fiber types in different muscles (Dubowitz and Brooke, 1973). Most muscles of humans (Dubowitz and Brooke, 1973) and the horse (Linholm and Piehl, 1974) (Aberle, 1976) are composed of 1/3 type I fibers, 1/3 type II_A fibers and 1/3 type II_B fibers. The type II fibers of sheep make up 60 to 95% of many of the muscles with an average of about 80% (Suzuki, 1971). Similar results were found in cattle (Holmes and Ashmore, 1972). Eighty-five % of the medial gastrocnemius of the cat was classified as type II fibers (Henneman and Olson, 1965). Different regions of muscle showed different proportions of red versus white fibers. In the guinea pig's vastus lateralis, because of grouping of red fibers, sampling of the entire muscle is necessary before the actual fiber proportions can be computed (Gillespie, et al, 1970).

Average diameters of normal human muscle fibers have been collected and muscle histograms and profiles developed to allow diagnosis of

atrophy, hypertrophy and muscle degeneration due to nervous disease or muscular dystrophy (Dubowitz and Brooke, 1973) (Dubowitz, 1968).

Work in animals has been developed for both research and diagnosis of animal disease. Type II hypotrophy has been described in the dog (Cardinet, 1969). Neurogenic atrophy has been confirmed in the crico-arytenoideus dorsalis muscle of the horse using the ATPase stain (Gunn, 1972) (Duncan, 1974). Type II_A and II_B fiber degeneration was present in the horse with acute rhabdomyolysis (Lindholm and Piehl, 1974). These techniques have been applied to the hereditary muscular dystrophies of the mouse (Fahimi and Roy, 1966) (Bajurz, 1965) and the chicken (Cosmos, 1966) (Cosmos and Butler, 1967) (Cardinet, 1967).

Developing muscle has been studied with histochemistry. No differentiation was found in skeletal muscle of the rat or mouse at birth (Dubowitz, 1968) but differentiation was seen nine days after birth. Based on the ATPase stain the rabbit and hamster showed differentiation into two fiber types at birth (Dubowitz, 1968). The cat showed differentiation into four fiber types using oxidative stains (Hammarberg, 1974). The guinea pig and human had differentiated early during gestation (Dubowitz, 1968). Human muscle shows complete differentiation into two fiber types at 28 weeks gestation with a distribution similar to that found in mature human muscle (Dubowitz, 1968). In general, maturity of animals at birth seemed to correlate with the differentiation of skeletal muscle (Dubowitz, 1968). In all cases the ATPase was the best histochemical stain distinguishing differentiation in the neonate. Sheep, cattle and pigs showed differentiation at birth with the oxidative enzymes (SDH and NADH-TR) though fibers in the sheep were sometimes difficult to separate with this stain (Ashmore and Doerr, 1971).

Table I

Histochemical Stain	Author	Species	I	II ⁻ A	II ⁻ B
NADH-TR	Ogata & Mori 1964	Fish, Bird, Mammals	Dense	Dense	Sparse
	Suzuki 1971	Sheep	Even Dist.	Subsarcolemma	"
	Dubowitz & Brooke 1973	Human	High	Medium	Low
ATPase 9.4	Padykula & Herman 1955	Rat	Light	Dark	Dark
4.6)	Brooke & Kaiser 1969	Human	Dark	Light	Medium
4.3)			Dark	Light	Light
Glycine 7.0	Tunnell 1975	Dog	Light	Medium	Dark
PAS	Dubowitz & Brooke 1973	Human	Light	Dark	Medium
Phosphorylase	Dubowitz & Brooke 1973	Human	Low	High	High
	Suzuki	Sheep	Low	High	High
Myoglobin	James 1974	Rabbit	--	High	Low
α Glycerol phosphate dehydroxylase	Dubowitz & Brooke 1973	Human	Low	Medium	High
SDH	Stein & Padykula 1952	Rat	Medium	High	Low
	Suzuki 1971	Sheep	Dense	Dense	Sparse
	Dubowitz & Brooke 1973	Human	High	Sarcolemma Medium	Low

TABLE II

Brooke & Kaiser (1970)	I	II _A	II _B
Ogata & Mori (1958)	Medium	Red	White
Engel (1962)	I	II	II
Stein & Padykula (1962)	B	C	A
Romanul (1964)	III	II	I
Padykula & Gauthier (1967)	Intermediate	Red	White
Gauthier (1969)	Red or Intermediate	Red	White
Samaha et al (1970)	B	C	A
Ashmore & Doerr (1971)	Beta Red	Alpha Red	Alpha White
Bernard et al (1971)	Slow Twitch Intermediate	Fast Twitch Red	Fast Twitch White
Suzuki (1971)	C	A	B
Peter (1972)	Slow Twitch Oxidative	Fast Twitch Oxidative- glycolytic	Fast Twitch Glycolytic
Khan et al (1973)	I Red	II Red	II White
Lindholm & Piehl (1974)	Slow Twitch	Fast Twitch High oxidative	Fast Twitch

HISTOCHEMICAL TECHNIQUES

Muscle Sample Collection

- Samples were collected from recently euthanatized sheep and from sheep kept as long as 72 hours at 4° C.
- Vastus intermedius was sampled by removing the entire medial belly.
- Vastus lateralis was sampled from the middle of the muscle removing a 1 x 1 x 2 cm. piece with the long edge parallel to the muscle fibers.
- Vastus medialis was sampled from the center of its medial surface removing a 1 x 1 x 2 cm. piece with the long edge running parallel to the muscle fibers, which are at a 30° angle to the surface of the muscle.
- Rectus femoris was sampled from the distal 1/3 of the muscle where a 1 x 1 x 2 cm. piece was removed with the long edge running parallel to the fibers.
- Samples were placed in gauze moistened in physiologic saline and stored for 1 to 12 hours at 4° C.

Quenching

- The muscle sample was cut in transverse sections less than 5 mm. thick when still cold from refrigeration.
- These 1 cm. square, 5 mm. thick sections were placed on mounting medium on 1 cm. squares of thick paper.
- Isopentane in a metal or glass beaker was lowered into liquid nitrogen in a Dewar flask.
- The isopentane was allowed to cool to -80° or the point at which the bottom of the isopentane was solidified.
- The muscle, attached to the paper, was quenched in the isopentane for 5 to 10 seconds and removed when convection currents at the surface of the muscle stopped or when the muscle started cracking.
- The muscle sample frozen to the paper was placed in a precooled vial at -30° C and sealed with a tight fitting cap.
- Muscle in the vial with label was stored at -70° C.

Frozen Sectioning of Muscle

- Muscle in vials was removed from the deep freeze and placed in the cryostat at -20° C.
- Muscle samples were attached to microtome chucks using mounting medium.
- The chuck was placed on the microtome.
- The face of the muscle was leveled until adequate area of muscle was being sectioned.
- 10 μ m sections were collected serially and picked up on warm (22° C) cover slips.
- The cover slips were placed in labeled Columbia jars and allowed to dry for 30 to 60 minutes.
- The sections were either stained immediately or the covers replaced in the Columbia jars which were then stored at -30° C for up to one week before staining.
- At the end of storage Columbia jars with cover slips were allowed to warm to room temperature before removing the cap to prevent condensation on the cover slips.

Adenosine Triphosphate Hydrolase (ATPase) with Tunell's pH 7
Preincubation

- Air dry frozen 10 um sections 30 minutes.
- Pre-incubation air dried sections for 5 minutes in 0.1 M glycine, 1% calcium chloride, 10% formaldehyde solution adjusted to pH of 7.
- Rinse with distilled water 5 times.
- Add 15.2 mg. Adenosine triphosphate (#A3127 Sigma):
 - 2 ml. 0.1 M Barbitol
 - 1 ml. 2% Calcium Chloride
 - 7 ml. Distilled H₂OMix and adjust to pH 9.4 with 1 N NaOH.
- Incubate sections in this medium at 37 degrees C for 2 hours.
- Wash with 1% CaCl₂ solution 3 to 4 times.
- 2% CoCl₂ solution for 3-5 minutes.
- Wash thoroughly 10 times over 5 minutes in distilled water.
- Place coverslips in 1% (NH₄)₂S solution for 30 seconds.
- Wash out (NH₄)₂S solution with tap water thoroughly and hold in water.
- Dehydrate in 95% and 100% alcohol.
- Clear in xylene and mount in synthetic medium.

ATPase with Acid Preincubation at pH 4.6, 4.45 and 4.3

- Air dry 10 um frozen sections for 30 minutes.
- Preincubate for 5 minutes in 0.1 M Na acetate buffers adjusted to pH 4.6, 4.45 or 4.3 using 1 N NaOH or 1 N Acetic Acid.
- Wash five times in distilled water.
- Place in ATPase incubating medium at pH 9.4 for 30 minutes.
- Follow procedure for normal ATPase development.

Nicotinamide Adenine Dinucleotide-Tetrazolium Reductase (NADH-TR)
(Suzuki, 1971^a)

--Air dry 10 um frozen sections for 30 minutes.

--Prepare 20 ml. of incubating medium from:

10 ml. of 0.2 M Tris buffer at pH 7.2

10 ml. of distilled water

10 mg. of Nitro-BT

5 mg. of NADH (reduced)

--Place cover slips in incubating medium for 90 minutes to develop blue stain.

--Place coverslips in 10% BNF for 10 minutes.

--Wash and hold in distilled water.

--Dehydrate in 95% and 100% alcohol.

--Clears in xylene and mount in synthetic medium.

Succinic Dehydrogenase (SDH) (Nachlas et al, 1957)

--Air dry 10 um frozen sections for 30 minutes.

--Prepare substrate from equal volumes of:

0.2 M sodium succinate

0.2 M phosphate buffer adjusted to 7.6 pH

--Mix:

10 ml. substrate

10 ml. distilled water

10 mg. Nitro BT

--Incubate coverslips for 1 hour at 30° C in medium.

--Wash with physiologic saline solution.

--Dehydrate in 95% and 100% alcohol.

--Clear in xylene and mount in synthetic medium.

Formulas for Solutions Used in Histochemistry

--0.1 M barbital, 20.62 g of Na barbital in one liter of distilled water.

Store in refrigerator.

--2% calcium chloride solution, 20 g CaCl_2 in one liter of distilled water. Store in refrigerator.

--1% calcium chloride solution, 10 g CaCl_2 in one liter distilled water.

--2% cobalt chloride, 20 g CoCl_2 in one liter of distilled water.

--0.1 M. glycine-calcium chloride Formal (Tunell's preincubation):

Dissolve 7.51 g glycine and 10 g CaCl_2 in distilled water to make 900 ml. Add 100 ml. 100% formalin and store at 4° C.

--1% (Dilute) $(\text{NH}_4)_2\text{S}$ solution 2 ml. of 20% $(\text{NH}_4)_2\text{S}$ in 100 ml. of distilled water. Make fresh before each use.

--0.2 M Acetate Buffer:

Solution A--Add 1.2 ml. glacial acetic acid to 100 ml. of distilled water.

Solution B--Na acetate 2.7 grams q.s. to 100 ml. with distilled water. Add solution A to solution B using pH meter to arrive at pH 4.3, 4.45 or 4.6.

--0.1 M Acetate Buffer:

Solution A--Add 0.6 ml. glacial acetic acid to 100 ml. of distilled water.

Solution B--Na acetate 1.35 g q.s. to 100 ml. with distilled water. Add solution A to solution B using pH meter to arrive at pH of 4.3, 4.45, 4.6 or 5.7.

--0.2 M Tris buffer:

Add 12.1 g Tris base and 1.863 ml. conc HCL to 400 ml. of distilled water q.s. to 500 ml. with distilled water.

Adjust to pH 7.2 with 1 N NAOH or 1 N HCL as needed.

--0.2 M Soldium Succinate:

Add 32.4 g Na succinate to one liter of distilled water.

--0.2 M Sodium phosphate buffer: 0.3312 g of NaH_2PO_4 and 4.7168 g NaH_2PO_4 to 100 ml. of distilled water.

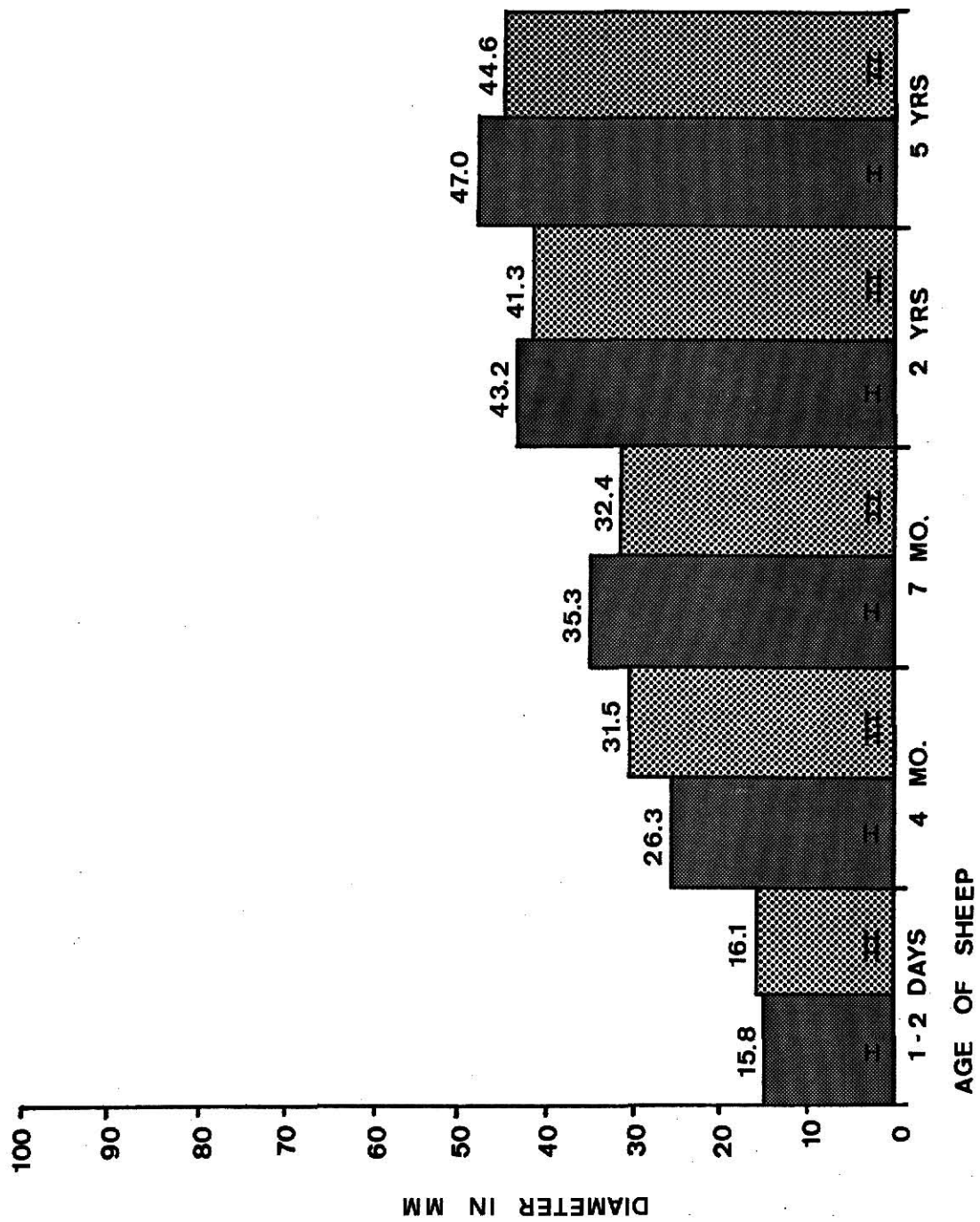
Photomicrography & Measuring of Muscle Fibers

- Stained serial sections of muscle were photomicrographed using a Leitz Orthomat with a 10x or 25x planapo objective resulting in a 33x magnification of the negative.
- Panatomic x film was used and developed in microdol - x.
- Photomicrographs were enlarged on 7x #3 Kodabromide paper with dektol producing a magnification of 231 x.
- Prints were fixed in hypo and washed and dried.
- Muscle fiber diameter was measured using a Zeiss particle size analyzer measuring the least diameter across the greater aspect of each fiber cross section.
- Each fiber type was measured and counted producing a total of about 200 fibers of all types in a sampling.

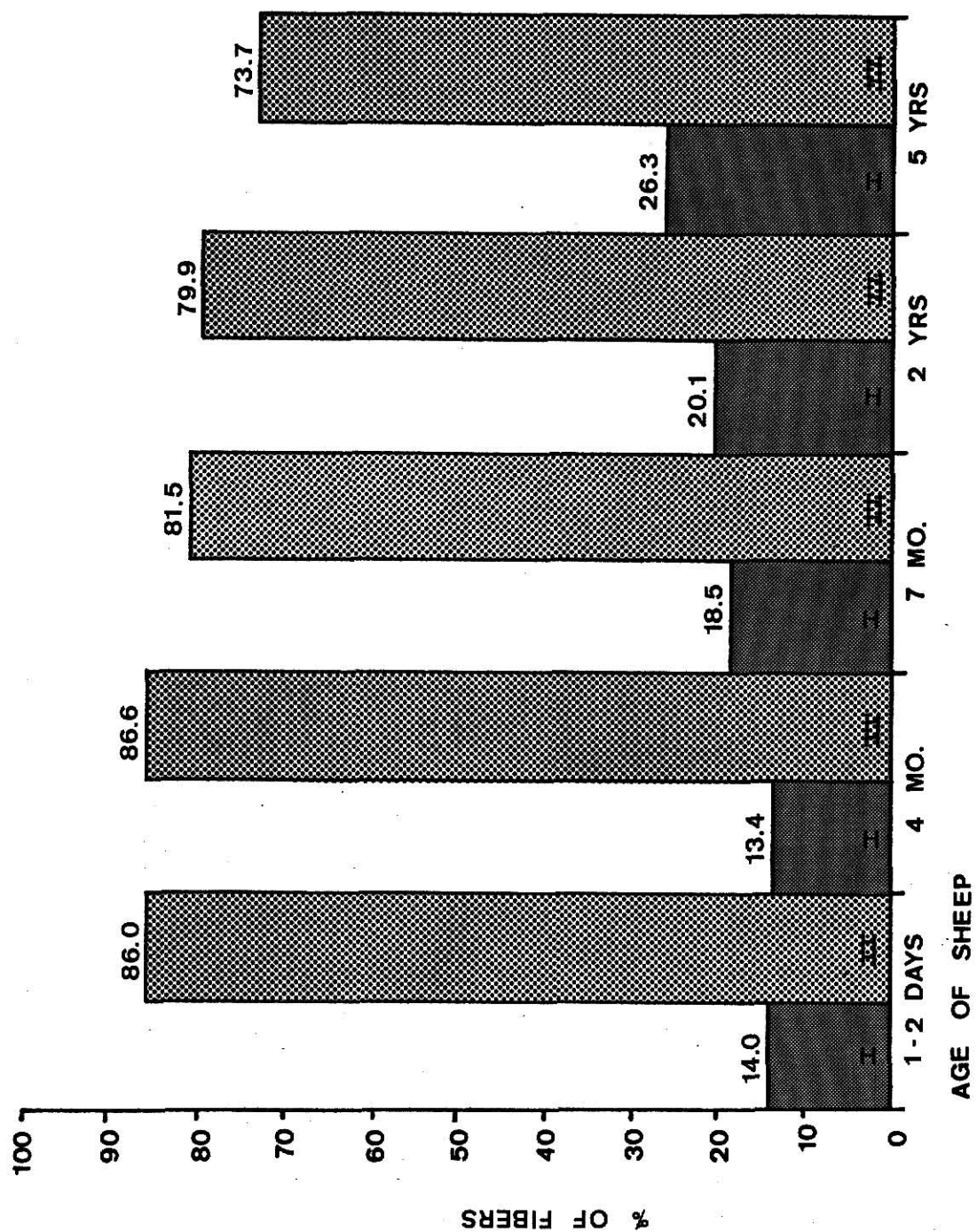
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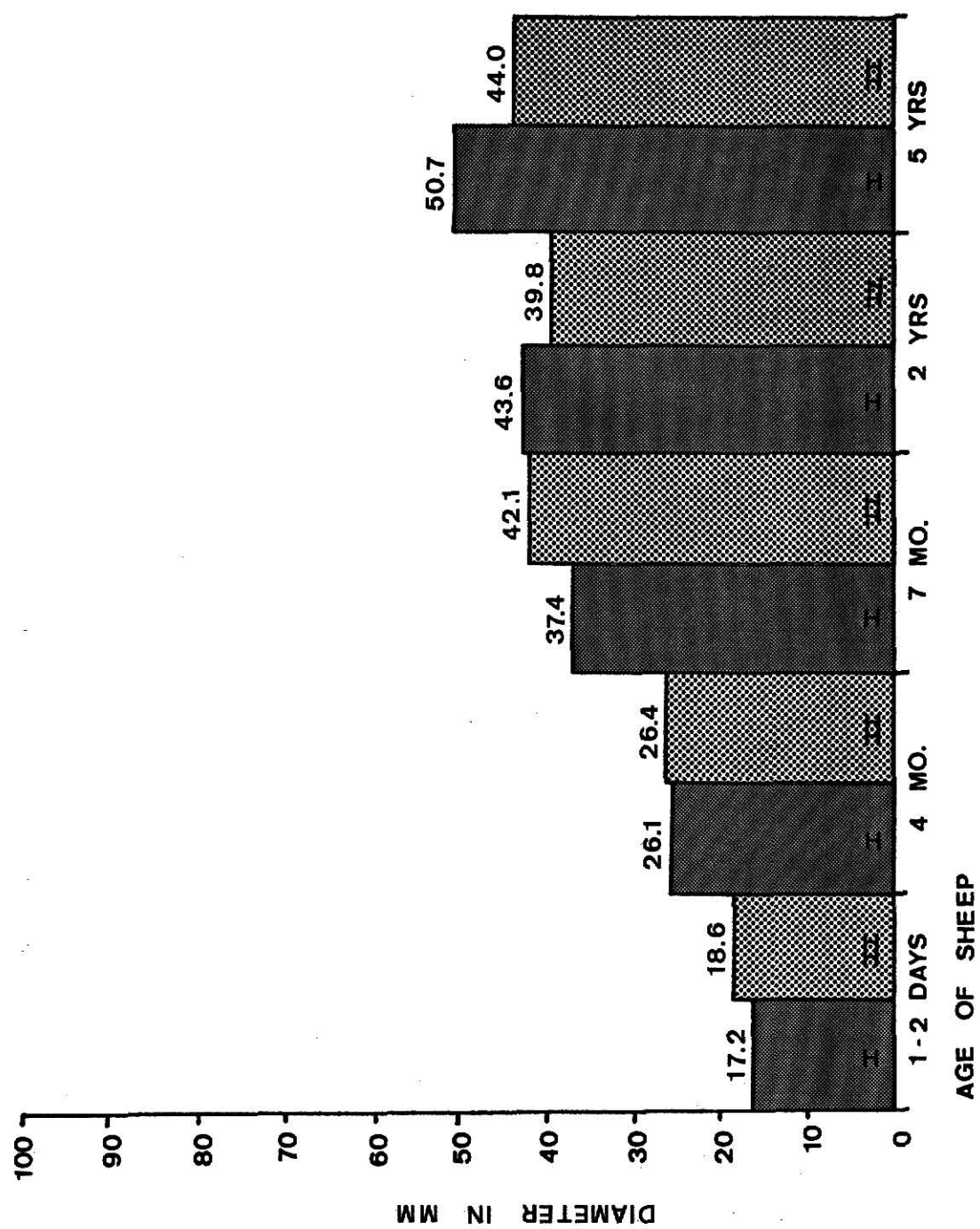
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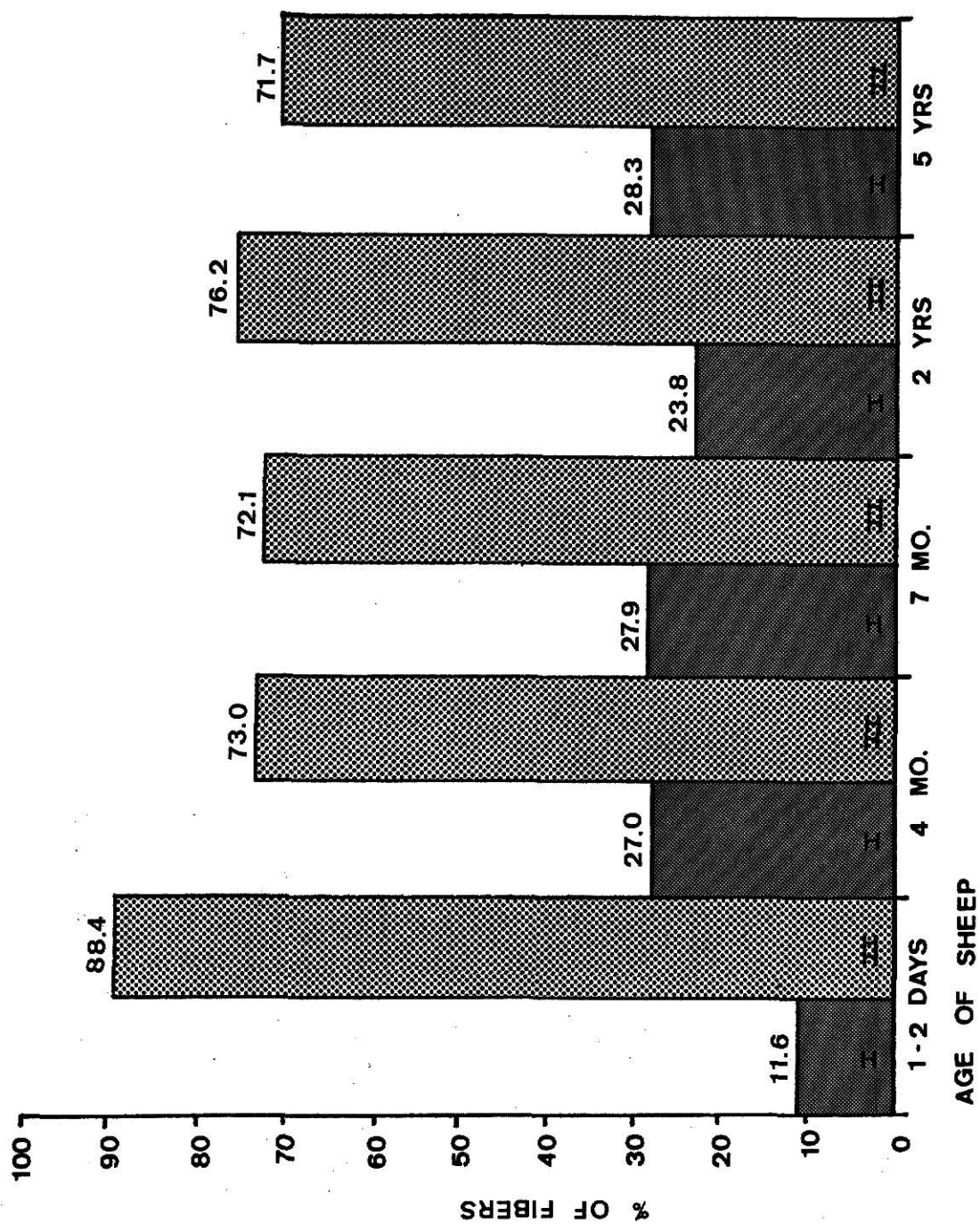
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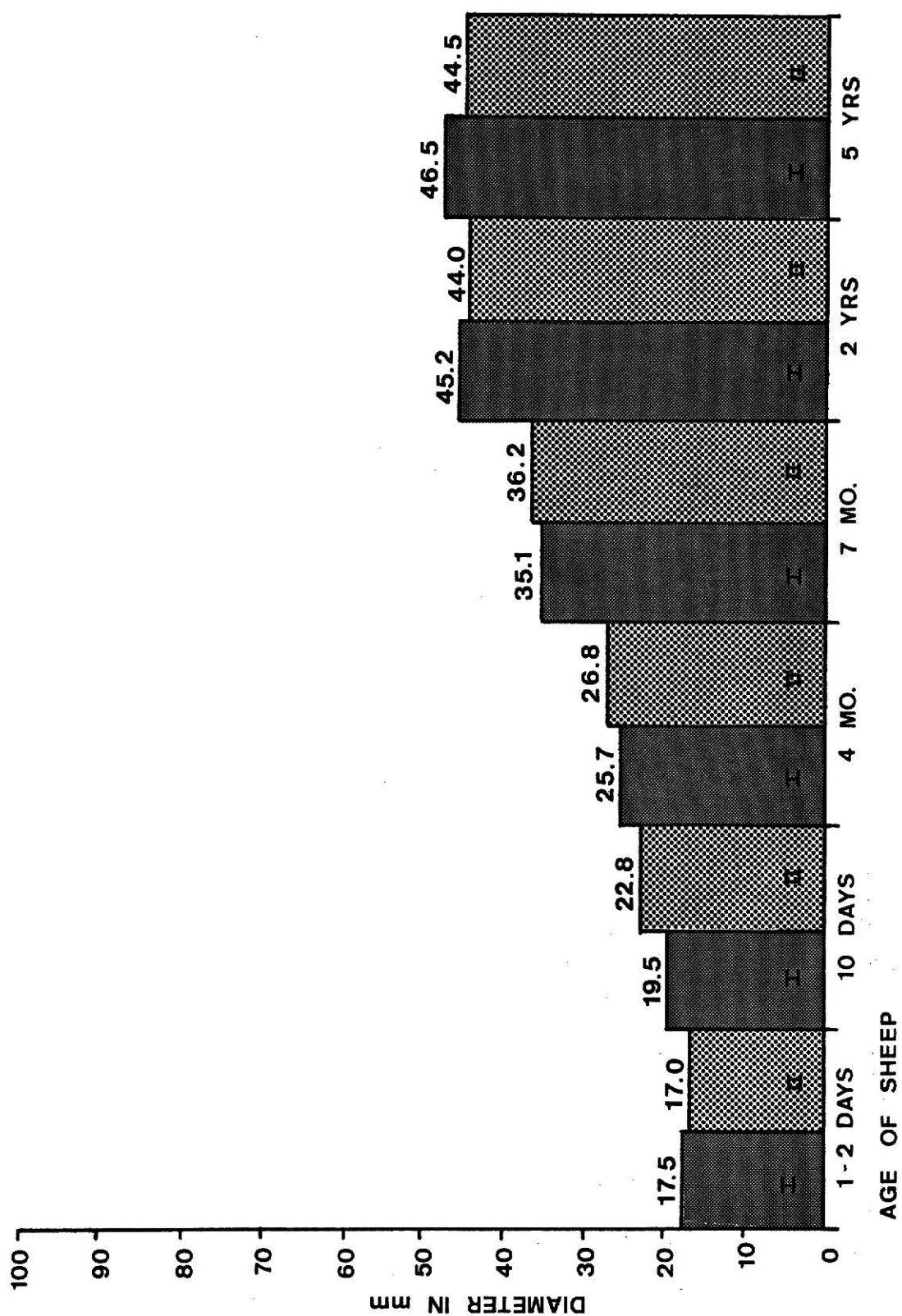
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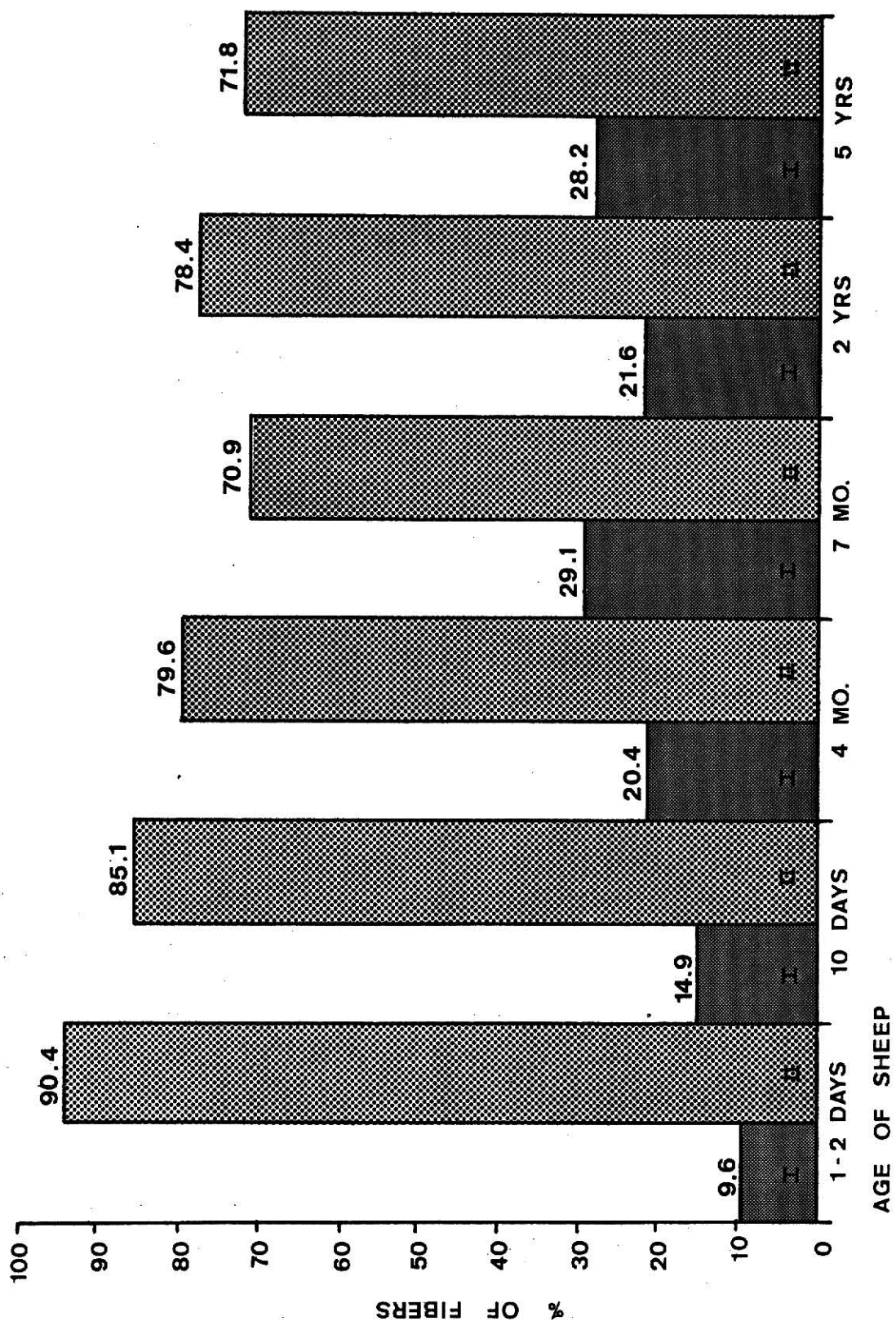
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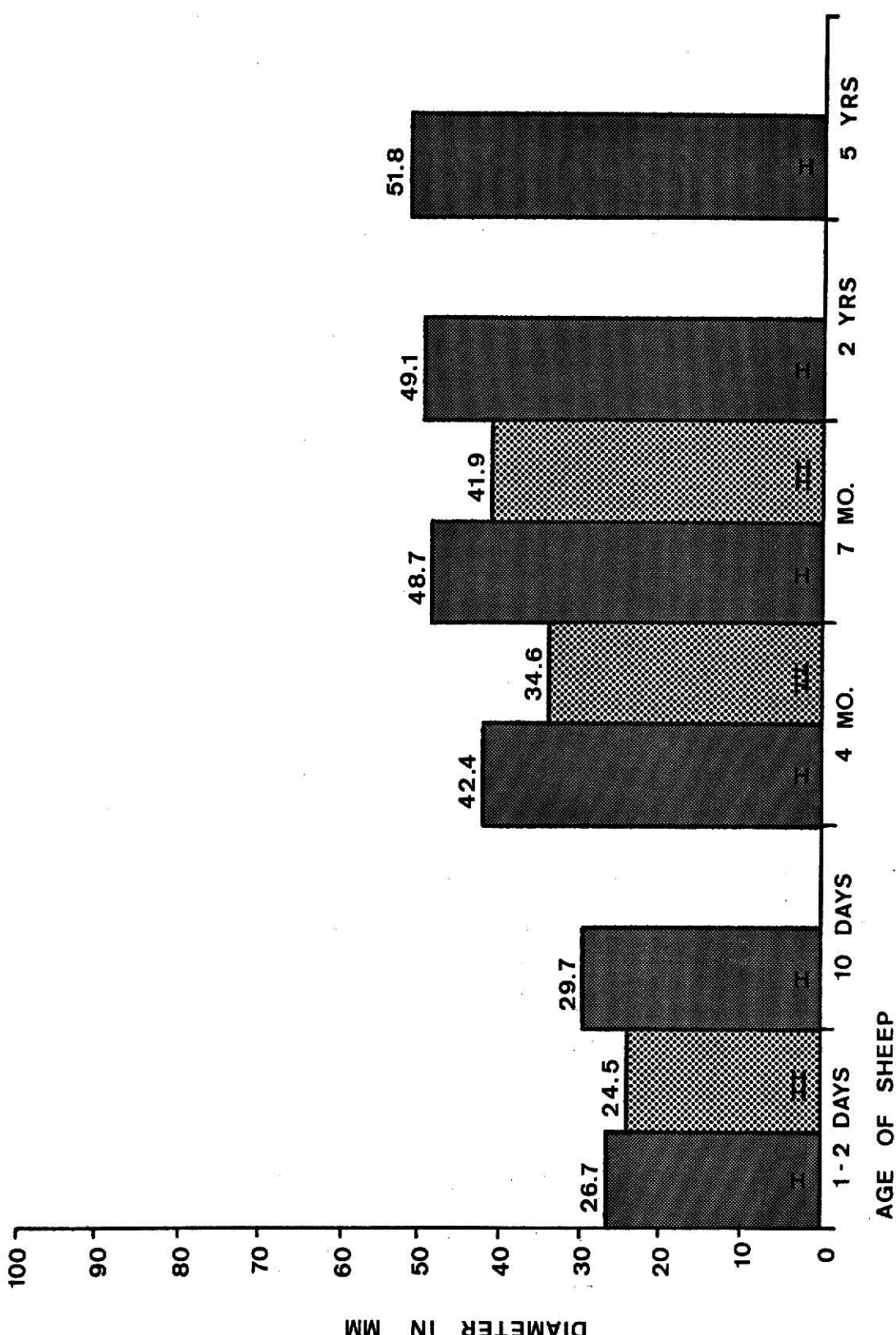
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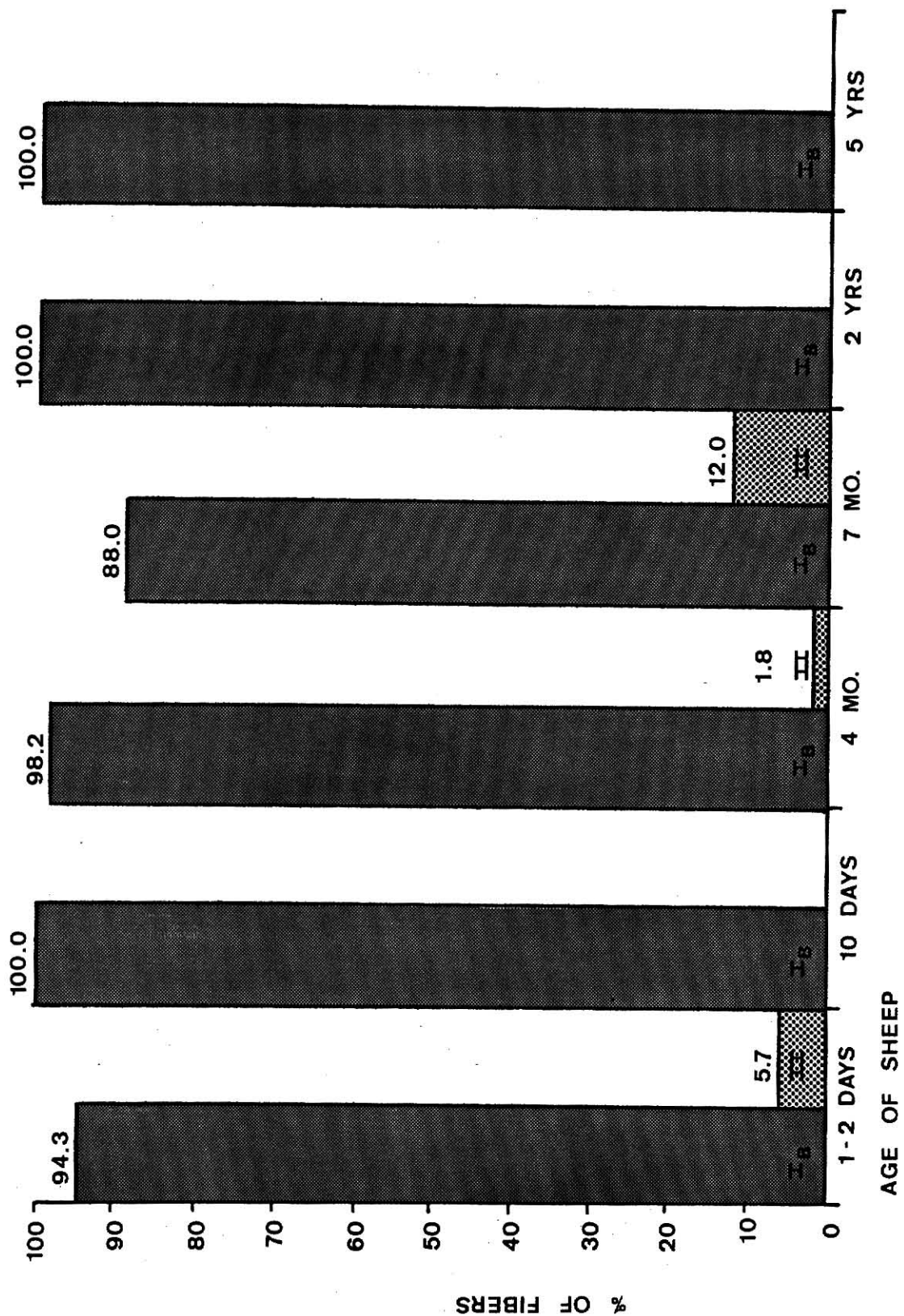
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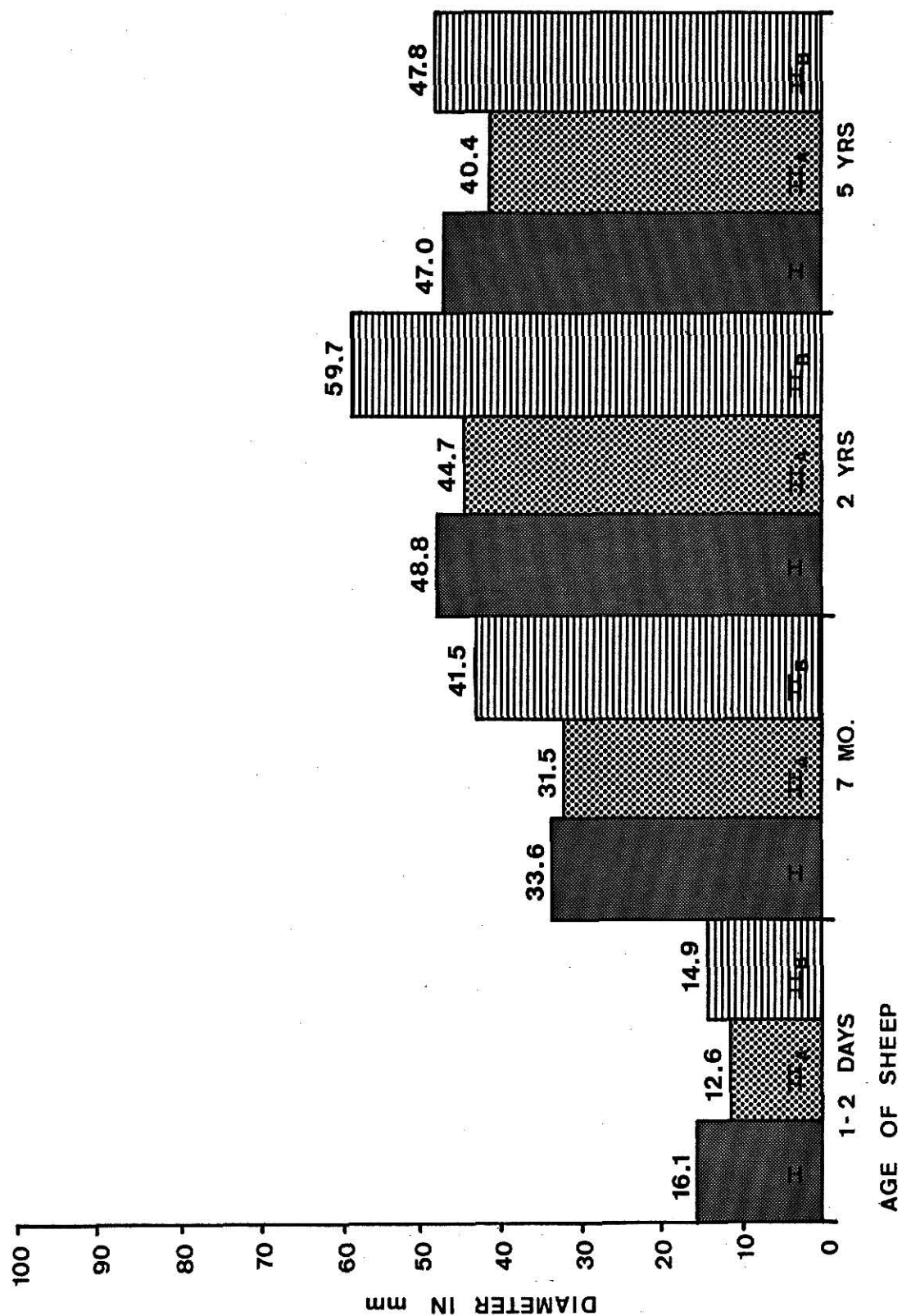
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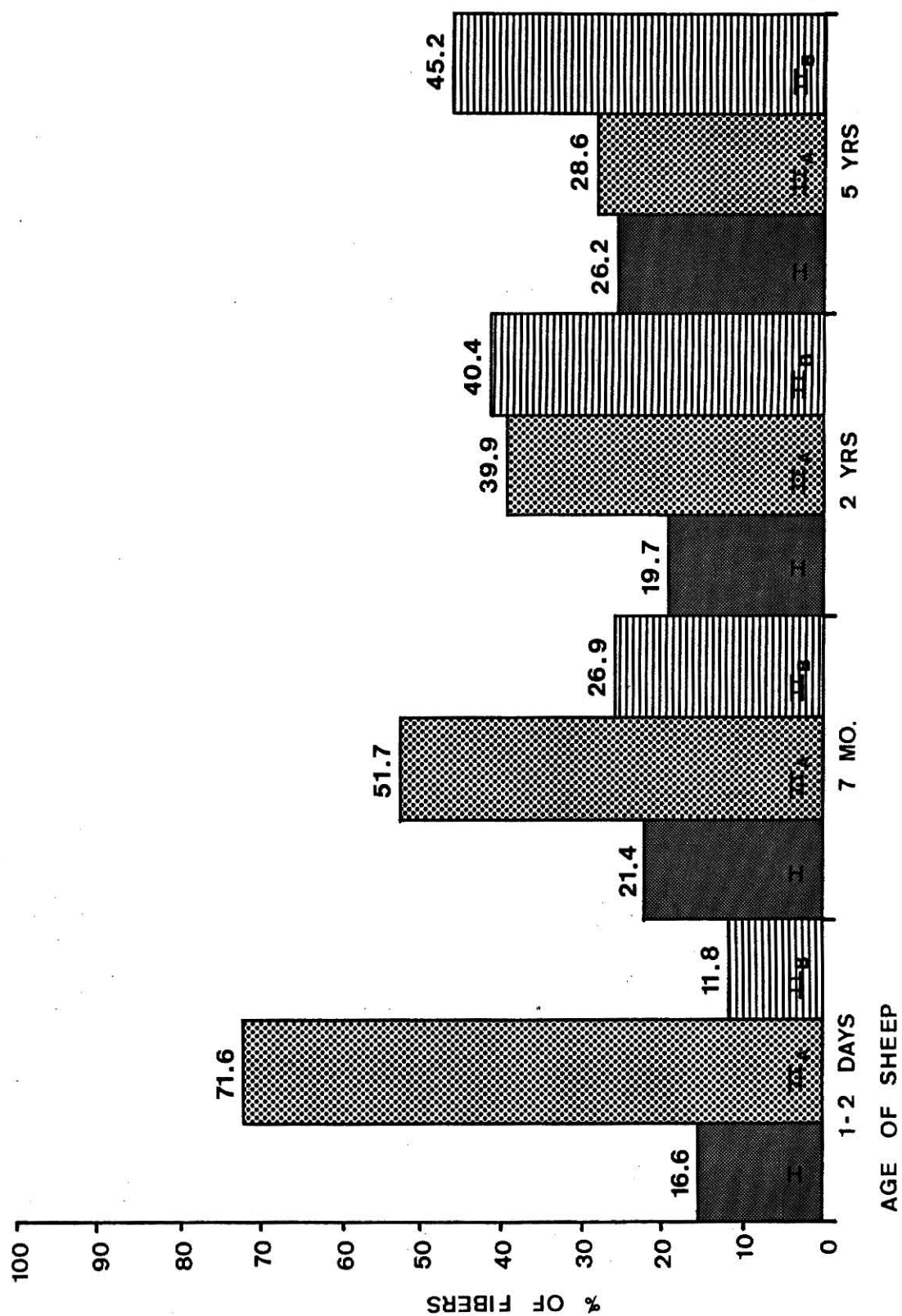
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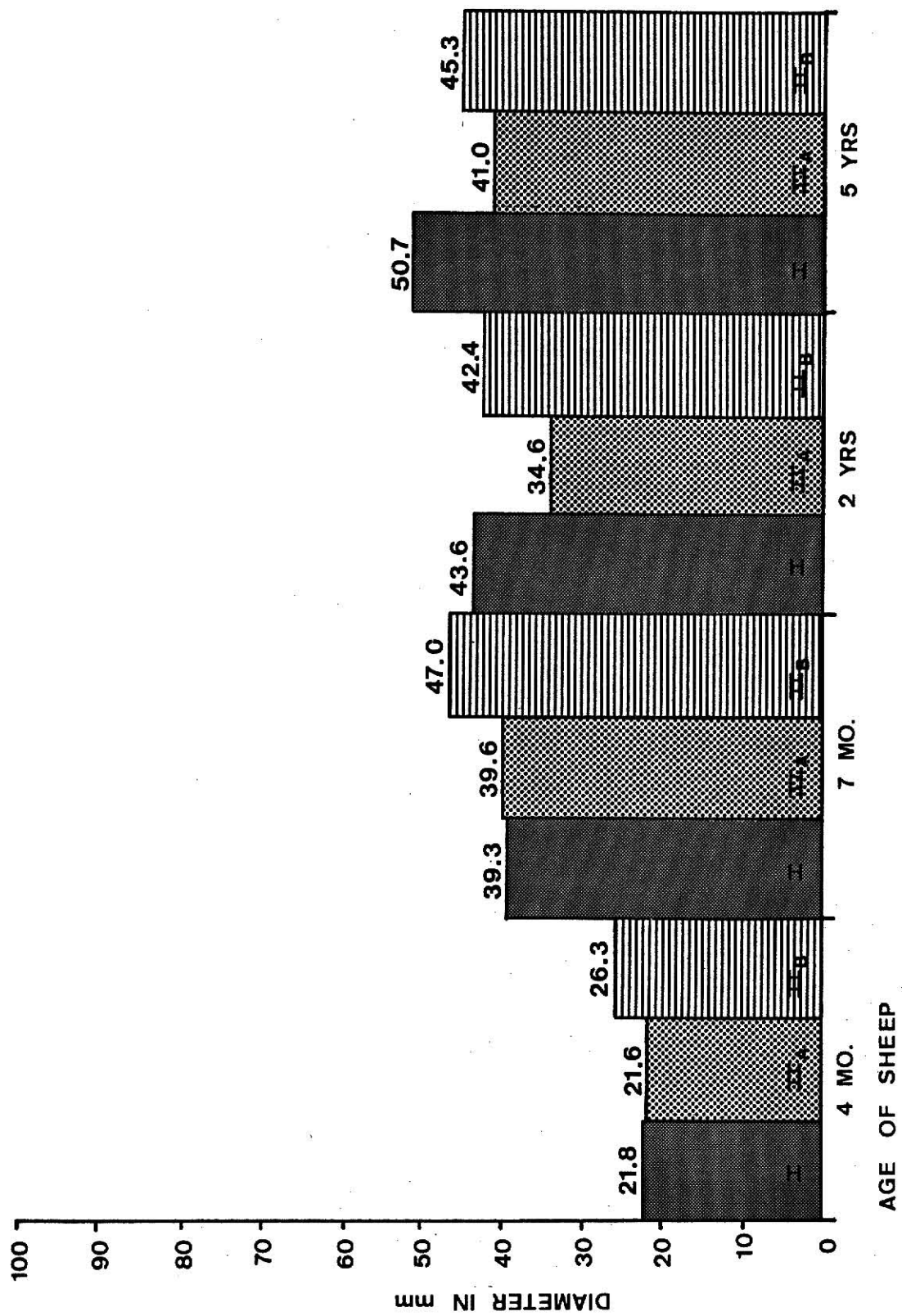
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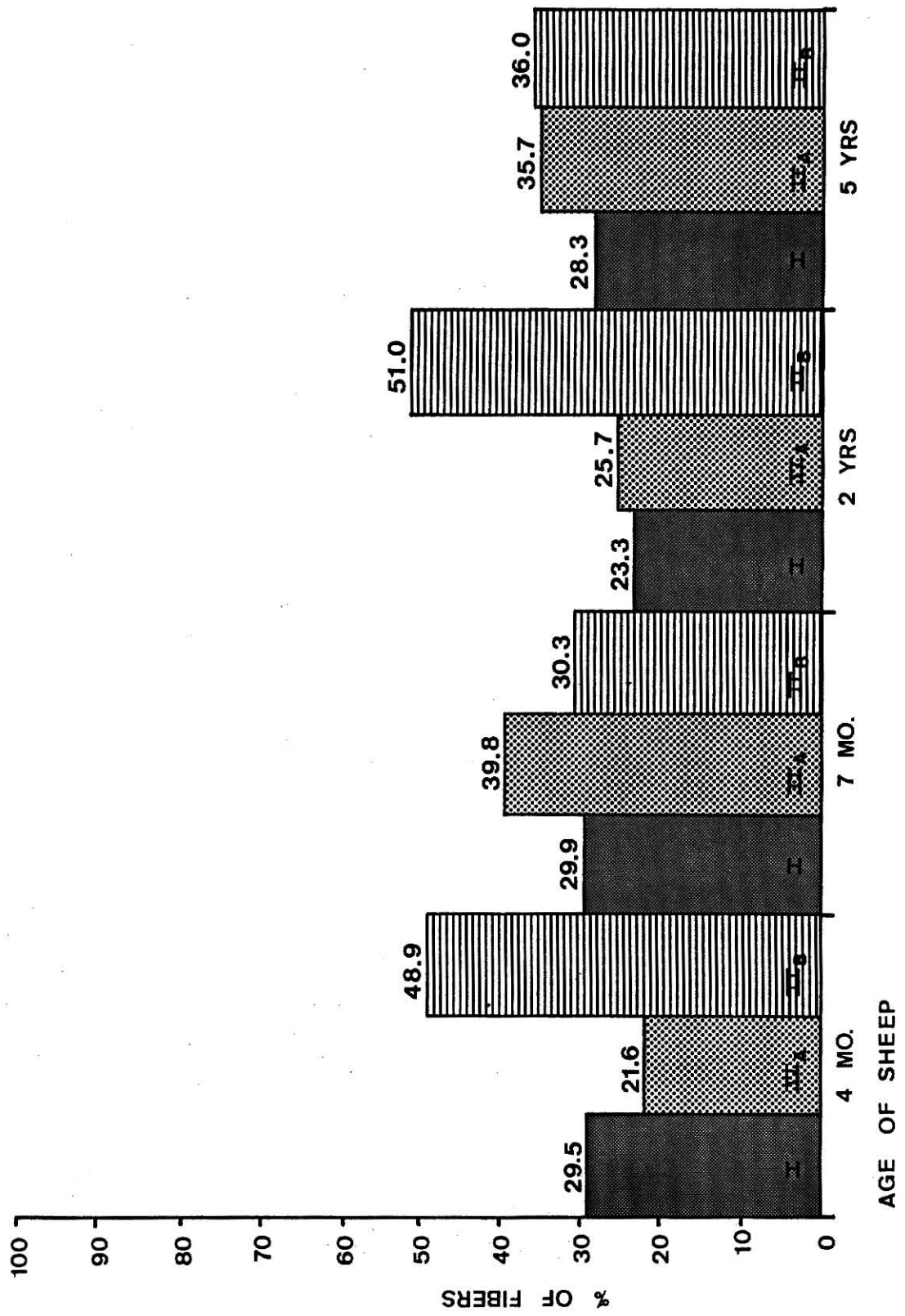
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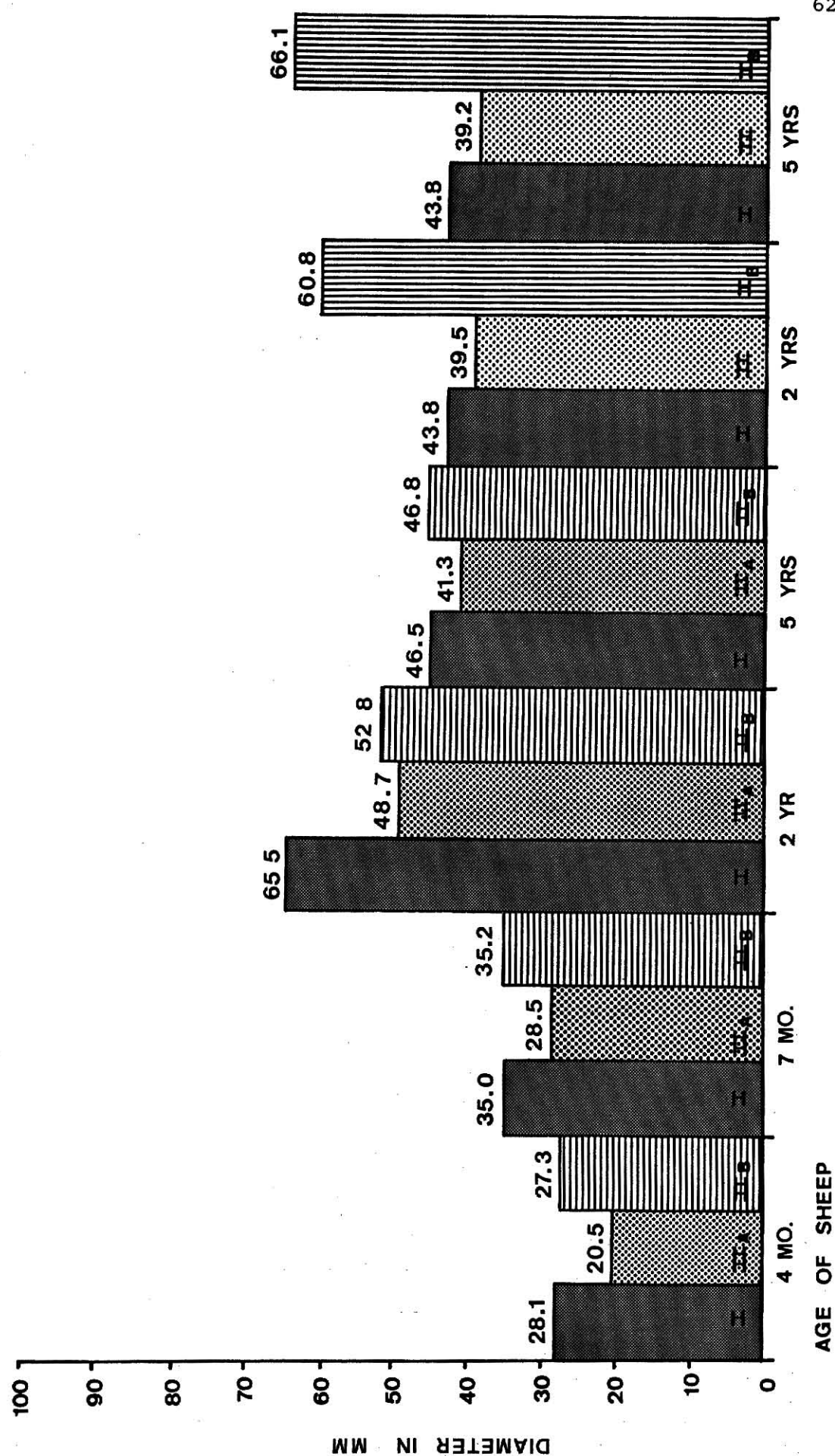
MUSCLE - VASTUS MEDIALIS



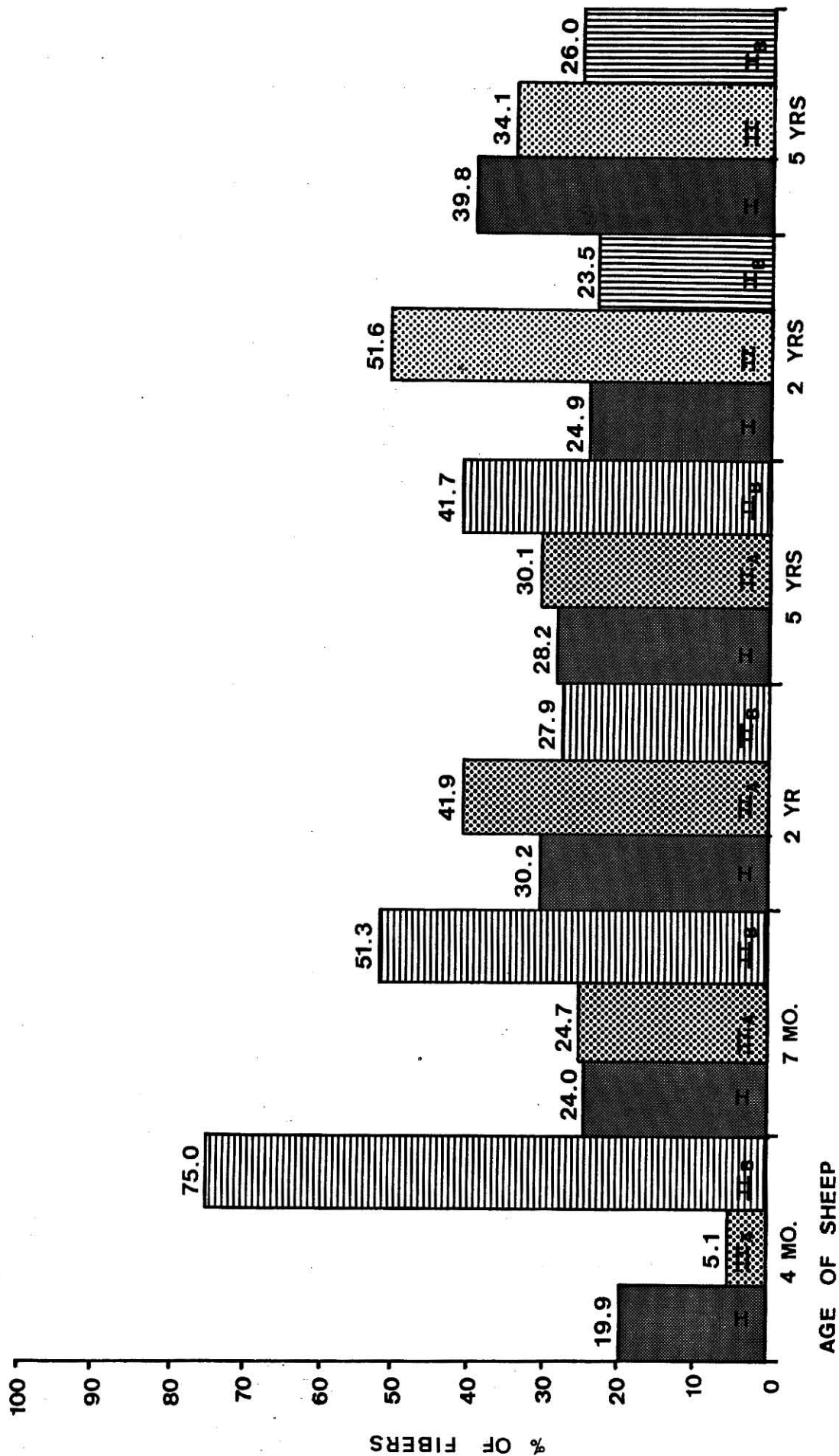
MUSCLE - VASTUS MEDIALIS



MUSCLE - RECTUS FEMORIS



MUSCLE - RECTUS FEMORIS



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DETERMINATION OF FIBER TYPE AND DIAMETER OF THE
OVINE QUADRICEPS MUSCLES AT DIFFERENT AGES

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ABSTRACT

Samples from the vastus lateralis (Vl), vastus medialis (Vm), vastus intermedius (Vi) and rectus femoris (Rf) from sheep 1 day to 5 years of age were quenched in isopentane at -90° C. Serial sections were stained with succinic dehydrogenase (SDH), nicotinamide adenine dinucleotide-tetrazolium reductase (NADH-TR) and myofibrillar adenosine triphosphate hydrolase (ATPase) and photomicrographs were taken, fiber types were classified, measured, and mean fiber diameter with standard deviation and proportion of each fiber type in the different muscles were determined.

The fiber proportion of the type I fiber increased from birth to 5 years of age from 9.6 to 28.6% in the Rf, 14.0 to 25.3% in the Vl and 14.1 to 28.3% in the Vm, the remaining proportion being made up of type II fibers. The Vi was always greater than 88% type I fibers and in many sheep the Vi was 100% type I fibers.

From birth to 5 years of age the mean fiber diameter of type I fibers increased from 17.5 to 46.5 μ m in the Rf, 15.8 to 47.0 μ m in the Vl, 15.6 to 50.7 μ m in the Vm and 26.7 to 51.8 μ m in the Vi. Type II fibers increased from 17.0 to 44.5 μ m in the Rf, 16.1 to 44.6 μ m in the Vl and 19.8 to 44.0 μ m in the Vm from birth to five years of age.

The type I fiber which almost entirely formed the Vi had strong diffuse NADH-TR staining and stained lighter than type I fiber with ATPase after pH 4.3 preincubation and slightly stronger than the type I fibers after glycine buffered calcium formal preincubation.